Studies on the Alkaline Amylase from an alkalophilic *Streptomyces* species NCL 716

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By

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Dedicated to

Bíbí

Aaí-Papa

Kamendra

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DECLARATION OF THE CANDIDATE

I declare that the thesis entitled **"Studies on the alkaline amylase from the alkalophilic *Streptomyces* strain NCL 716"**submitted for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Dr. Lalitha S Kumar** and has not formed the basis for the award of any degree, diploma, associate ship, fellowship, titles in this or any other University or other institute of Higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Trupti M Jamalpure

(Research Scholar)

LIST OF ABBREVIATIONS USED

Amy Strplo 716	*Streptomyces lonarensis* strain NCL 716 Amylase
ANS	1-anilino-8-naphthalenesulfonate
Arg	arginine
Asp	aspartic acid
AP1	adaptor primer 1
AP2	adaptor primer 2
CD	circular dichroism
Cys	Cysteine
Da	dalton
DEAE	di ethyl amino ethyl
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNSA	Di-nitro Salicylic Acid
DTNB	2, 2'-dithiobisnitrobenzoic acid
GdnHCl	guanidium hydrochloride
Glc	glucose
Gly	glycine
His	histidine
Hour	h
HPLC	high pressure liquid chromatography
IUPAC	international union of pure and applied chemistry
Km	Michaelis-Menten constant
Ksv	Stern-Volmer quenching constant
LB	Luria Bertani Broth
Lys	Lysine
Maltooligosaccharides	MOS
Maltotriose	G3
Maltotetraose	G4
Maltopentaose	G5
Maltohexaose	G6
Maltoheptaose	G7
MGYP	Malt extract Glucose Yeast extract Peptone agar
Minutes	min
MRE	mean residue ellipticity
$NaBH_4$	sodium borohydride
NAI	N-acetyl imidazole
NBS	N-bromo succinimide
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDB	protein data bank
pHMB	p-hydroxymercurybenzoate
pKa	acid dissociation constant
Pro	Proline
PSA	Potato Starch Agar
rpm	revolutions per minute

RMSD, SD	root mean square deviation, standard deviation
S	substrate
SDS	sodium dodecyl sulphate
Trp	tryptophan
Tyr	tyrosine
V, V_{max}	rate of an enzyme-catalyzed reaction, maximum reaction rate

Abstract

Title of thesis: "Studies on alkaline amylase from alkalophilic *Streptomyces* species strain NCL 716"

The theme of the present work is the characterisation of the alkaline α-amylase that is secreted by the alkalophilic *Streptomyces* strain NCL 716. Complimentary studies also include characterisation of the strain, fermentation studies to optimize enzyme yield and cloning, sequence analysis and homology modeling of the partial gene encoding the α-amylase.

This thesis is organised into the following six chapters:

Chapter 1: Introduction

Chapter 2: Characterization of the *Streptomyces* strain NCL 716

Chapter 3: Optimization of fermentation for the production of the alkaline alpha-amylase using SVR and MJWA

Chapter 4: Purification and characterization of the alpha amylase from *Streptomyces* strain NCL 716

Chapter 5: Cloning of the gene encoding the alpha-amylase from the *Streptomyces* strain NCL 716

Chapter 6: Discussions

CHAPTER I: Introduction

This introductory chapter gives a comprehensive literature survey of microbial amylases with an emphasis on α-amylases from alkalophiles, maltooligosaccharides producing amylases and α-amylases from *Streptomyces*.

A number of α-amylases from a wide variety of microbes have been isolated, purified and characterised and have been extensively covered in many reviews. However, most of these α-amylases are active at acidic or neutral pH. Pioneering investigations in Japan by Horikoshi and his associates have contributed to significant advances in the

understanding of alkalophiles and their enzymes. Alkaline α-amylases have been mainly reported from the genus *Bacillus.* Search for α-amylases active and stable in alkaline pH conditions and higher temperatures has intensified, since such enzymes would be suitable for the detergent, leather and paper and pulp industry where the operating pH range is above pH 8 and the temperature range is 40^0C - 60^0C. The few α-amylases reported from the genus *Streptomyces* have acidic or neutral pH optimum. To date there are only four reports of alkaline amylases from this genus, two of which are patented.

Specific malto-oligosaccharide producing enzymes have been mainly identified in bacteria. In the genus *Streptomyces*, there is one report of a neutrophilic strain and two reports of alkalophilic strains producing maltotriose. There is only one report of an acidophilic strain producing maltotetraose.

The alkaline amylases, the maltooligosaccharides producing amylases as well as the amylases from *Streptomyces* have been well characterised. The genes encoding several of these amylases have also been cloned and expressed in different hosts

This chapter also discusses the important properties of microbial α-amylases that have been deciphered using techniques such as chemical modification, X-ray crystallography, Fluorescence and Circular Dichroism.

The last section of this chapter describes the various applications of microbial α-amylases.

CHAPTER II: **Characterization of the actinomycete strain NCL 716.**

An actinomycete strain NCL 716 was isolated from the soil sample of Lonar Lake in Buldhana district, Maharashtra State. 16s rDNA analysis confirmed that this strain belongs to the genus Streptomyces. Hence it was named *Streptomyces lonarensis* strain 716 and was given the GenBank accession number FJ 919811. This chapter describes the characterisation of this strain using a polyphasic approach ie rDNA analysis, classical and chemotaxonomical approaches. This strain was found to be a facultative alkalophile which grows in the pH range of 8 to 11 and at 28^0C to 30^0C. This is the sixth report of an alkalophilic *Streptomyces* strain. An important

characteristic of this strain is that it secretes an alkaline α-amylase. Molecular characterisation of this α-amylase is the central theme of this thesis.

CHAPTER III : Optimization of fermentation for the production of the alkaline alpha-amylase using SVR and MJWA

This chapter discusses the optimization of a cost-effective medium for maximizing the yield of the amylase from the *Streptomyces lonarensis* strain 716, using a combination of Plackett-Burman, support vector regression (SVR) technique and multicanonical jump walk annealing algorithm (MJWA). The amylase yield obtained by using these approaches was 297 U/ml. As described in Chapter IV, this α-amylase hydrolyses starch to give a mixture of maltotriose, maltotetraose and maltose. The optimised enzyme activity compares well with the activity of reported amylases producing maltooligosaccharides.

CHAPTER IV: Purification and characterization of alkaline alpha amylase from *Streptomyces* strain NCL 716

This chapter describes the purification and characterisation of the α-amylase from *Streptomyces lonarensis* strain NCL 716. The enzyme was purified using a combination of ammonium sulfate precipitation (90%) and preparative PAGE (5%). Properties of the enzyme have been studied using conventional techniques as well as by chemical modification of different amino acid residues, fluorescence and circular dichroism.

CHAPTER V: Cloning of the gene encoding the α-amylase from the *Streptomyces* strain NCL 716.

This chapter describes the cloning and sequence analysis of the partial gene encoding the alkaline α-amylase from the *Streptomyces lonarensis* strain NCL 716 and also homology modeling of the translated protein. The gene sequence has been submitted to the GenBank with the accession number ADZ45287. This chapter also describes different protocols that were used to standardise the extraction of DNA from this strain and their

relative merits and demerits. A preliminary study on the methylation status of the genomic DNA using isoschizomer pairs of restriction enzymes has also been included.

CHAPTER VII: Discussion

This chapter highlights the significant findings of the present work.

Chapter 1

Introduction

Summary

Amylases comprise a group of enzymes which hydrolyze starch to produce products ranging from oligo-saccharides to limit dextrins. Though starch is a common substrate, the individual members differ in their specificity of action on starch. Amylases are distributed throughout the plant, animal and microbial kingdoms, but the microbial amylases have found wide biotechnological and industrial application because (i) they are more stable than their plant and animal counterparts (ii) they can be easily manipulated to suit various industrial need and (iii) their bulk production is economical. Amylases constitute almost 25% of the enzyme market because the degradation of starch by amylases is one of the most important enzymatic reaction having wide industrial applications. The focus of this thesis is an alkaline malto-oligosaccharides producing α-amylase from an alkalophilic *Streptomyces* strain named as *Streptomyces lonarensis* strain NCL 716. Hence this introductory chapter presents a comprehensive review on the microbial α-amylases with emphasis on alkaline α-amylases, malto-oligosaccharides forming α-amylases and α-amylases produced by Streptomyces. In addition, an attempt has also been made to present (i) a broad literature overview of the properties of microbial α-amylases as deciphered from structure-function relationship, and (ii) important application of microbial α-amylases.

Introduction

I. Starch degrading enzymes

The degradation of starch by amylases is one of the most important enzymatic reaction having wide industrial applications (Gupta *et al*, 2003). These applications include starch liquefaction and saccharification in the starch industry, in textile industry for de-sizing, in detergent industry for removal of starchy stains, in baking industry - to improve bread softness and volume, in flour adjustment, as viscosity providers, as flavor carriers, and as crystallization inhibitors, in the beverage industry for juice treatment and production of low calorie drinks, in the production of glucose and fructose syrups, in production of fuel ethanol from starches and in the paper and pulp industry for starch coating, de-inking and drainage improvement (Nigam and Singh, 1995; Nielsen and Borchert, 2000; Kirk *et al*,2002). The spectrum of application of amylases has extended to other fields such as clinical, medical and analytical chemistry (Pandey, 2000; Aiyer, 2005).

Amylases are glycosyl hydrolases (GHs) as they cleave the glycosidic linkages between the glucose units in starch. The term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4 glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Bernfeld,1955, Fisher and Stein, 1960). A number of enzymes associated with degradation of starch and related polysaccharides structures have been reported and studied (Boyer and Ingle, 1972; Griffin and Fogarty, 1973; Fogarty and Griffin, 1975). The most common classification of the glycosyl hydrolases has been to group them into different GH families, based on their amino acid sequence and structural similarities (CAZyserver http://www.cazy.org/). The starch degrading enzymes are found mainly in GH family 13, 14 and 15 (Henrissat, 1991; Henrissat and Davies, 1997; Coutinho and Henrissat, 1999).

Substrate for amylase

Starch is a very well known, conventional and a natural substrate for amylase. Starch is a major carbohydrate reserve of all higher plants and starches are commercially produced from the seeds of plants, like corn, wheat, sorghum or rice, from the tubers and roots of plants such as cassava, potato, arrowroot and the pith of sago palm. Corn is the major commercial source of starch from which it is extracted by a wet milling process (Berkhout, 1976). In few cases, it accounts for as high as 70% of the undried plant material and occurs in the form of water insoluble granules (Aiyer, 2005). Starch is synthesized in plastids and is found in leaves as a storage compound. It

is also synthesized in amyloplasts in tubers, seeds and roots. Starch is a long term storage compound. The shape and diameter of these granules depends upon the botanical origin. The granule size ranges from 2-30 μm (maize starch) to 5-100 μm (potato starch) (Robyt, 1998). When starch is heated in water the hydrogen bonds holding the granules weaken and this permits the molecules of starch to swell and gelatinize, resulting in a paste or in a dispersed form depending on the concentration of the polysaccharides.

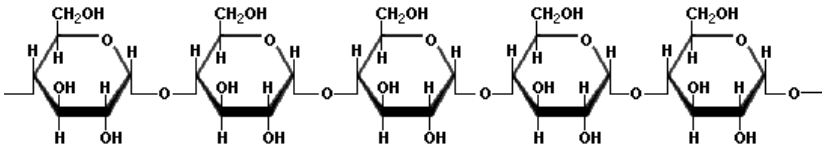
Structure of starch

Starch is a heterogeneous polysaccharide composed of two high molecular weight polymers i.e amylose and amylopectin which differ in their structure and physical properties.

a) Amylose

Amylose is a linear polymer consisting of upto 6000 glucose units linked via α 1-4 glycosidic bonds. The number of glucose residues, defined by the term DP (degree of polymerization), varies with the origin (Myers and Meyrs, 2000). Amylose from potato or tapioca starch has a DP of 1000-6000 while amylose from maize or wheat has a DP varying between 200 and 1200 residues. The average amylose content in starch varies anywhere between 0 to 75 %, but a typical value is 20-25 %. Since, amylose is a linear molecule it tends to precipitate spontaneously. This is because the linear chains align themselves by hydrogen bonding resulting in aggregation. Precipitated amylose is soluble only in alcoholic solutions.

Structure of amylose



b) Amylopectin

Amylopectin accounts for 75 to 85 % of most starches. Its molecular weight is approximately in the range of 10^7 to 10^8 and it has a branched structure composed of chains of about 20-25 α 1-6 linked D-glucose residues. Amylopectin may contain 4 to 5 % of ά 1-6 D-glucosidic bonds. The complete amylopectin molecule contains on an average of about 20,00,000 glucose units, thereby being one of the largest molecules in nature.The most commonly accepted modular structure of amylopectin is the

cluster model, in which the side chains are ordered in clusters on the longer backbone chain (Myers and Meyrs, 2000). The starch granules are organized into amorphous and crystalline regions. In tubers, starch is mainly composed of amylopectin (crystalline) while amylose is present in amorphous regions.

The two polymers differ in their structure and physical properties and the differences are summarized in **Table 1.1.** (Aiyer, 2005)

Structure of amylopectin

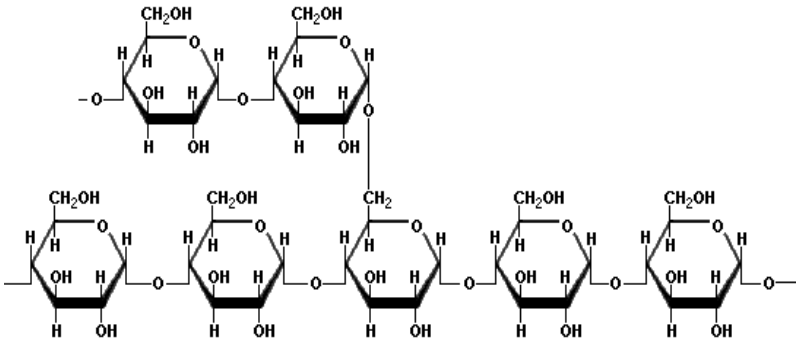


Table 1.1: Properities of amylose and amylopectin

Properties	Amylose	Amylopectin
Basic Structure	Essentially linear	Branched
Degree of polymerization	C.10^3	C. 10^4 - 10^5
Average chain length	C.10^3	C. 20 – 25
λ max for iodine complex	650 nm	550 nm
Stability in aqueous solution	Precipitates sponta-neously	Stable

The GH family 13 is known as the α-amylase family and the important starch degrading enzymes included in this family are α-amylases (EC 3.2.1.1), maltogenic α-amylase (EC 3.2.1.133), pullulanase (EC 3.2.1.41), iso-amylase (EC 3.2.1.68), maltotriose-forming α-amylase (EC 3.2.1.116), maltotetraose - forming α-amylase (EC 3.2.1.60) and maltohexaose forming α-amylase (EC 3.2.1.98) (Coutinho and Henrissat, 1999; CAZy server http://www.cazy.org/). α-amylases (EC 3.2.1.1) (1,4- α-D-glucan glucanohydrolase, glycogenase, α amylase, α-amylase, endoamylase, Taka-amylase A) hydrolyzes the α-1,4 glucosidic linkages in starch and related substrates in an endofa-

shion, but the α-1, 6 glycosidic linkages on branched polymers are not attacked (Bolton *et al*, 1997). Amylopectin is comparatively more stable due to its branched nature and does not form compact aggregates. Being endo in action, α amylases affect a rapid decrease in iodine staining power with a simultaneous rapid decrease in viscosity of starch. Hydrolysis of amylose by α-amylases results in the production of oligosaccharides of different chain length, maltotriose and maltose. Maltotriose being a poor substrate is hydrolyzed slowly. Hydrolysis of amylopectin by α-amylase also yields glucose, maltose and α-limit dextrins. The various α-amylases produce different α-limit dextrins. α-amylases which produce free sugars are termed as saccharogenic and those that liquefy starch without producing free sugars are known as starch liquefying enzymes.

Maltogenic α-amylase (EC 3.2.1.133) (1,4-α-D-glucan α-maltohydrolase, 4-α-D-glucan α-maltohydrolase) acts on the (1→4)-α-D-glucosidic linkages in starch and related polysaccharides and oligosaccharides to remove successive α-maltose residues from the non-reducing ends of the chains.

Pullulanase (EC 3.2.1.41) (pullulan 6-α-glucanohydrolase, amylopectin 6-glucanohydrolase, bacterial debranching enzyme, debranching enzyme, α-dextrin endo-1,6-α-glucosidase, R-enzyme, pullulan α-1,6-glucanohydrolase) hydrolyses the (1→6)-α-D-glucosidic linkages in pullulan, amylopectin and glycogen, and in the α- and β-limit dextrins of amylopectin and glycogen leaving long linear polysaccharides. Pullulan is a polysaccharide having a repeat unit of maltose triose that is α-1,6 linked.

Isoamylase (EC 3.2.1.68) (glycogen α-1,6-glucanohydrolase, debranching enzyme) hydrolyses the (1→6)-α-D-glucosidic branch linkages in glycogen, amylopectin and their β-limit dextrins. It differs from pullulanase (EC 3.2.1.41) by its inability to hydrolyse pullulan, and by limited action on α-limit dextrins.

Maltotriose-forming α-amylase (EC 3.2.1.116) (4-α-D-glucan maltotriohydrolase, glucan 1,4-α-maltotriohydrolase, exo-maltotriohydrolase, maltotriohydrolase, 1,4-α-D-glucan maltotriohydrolase) hydrolyses the (1→4)-α-D-glucosidic linkages in amylaceous polysaccharides, to remove successive maltotriose residues from the non-reducing chain ends.

Maltotetraose forming α-amylase (EC 3.2.1.60) (4-α-D-glucan maltotetraohydrolase, glucan 1,4-α-maltotetraohydrolase, exo-maltotetraohydrolase, 1,4-α-D-glucan maltotetraohydrolase) hydrolyses the (1→4)-α-D-glucosidic linkages in amylaceous polysaccharides, to remove successive maltotetraose residues from the non-reducing chain ends.

Maltohexaose forming α-amylase (EC 3.2.1.98) (4-α-D-glucan maltohexaohydrolase, exo-maltohexaohydrolase, 1,4-α-D-glucan maltohexaohydrolase) hydrolyses the (1→4)-α-D-glucosidic linkages in amylaceous polysaccharides, to remove successive maltohexaose residues from the non-reducing chain ends.

The α-amylases of family GH 13 have a common three-dimensional structure comprising of three structural domains A, B and C .The A domain is the catalytic domain and has a $(\beta/\alpha)_8$ barrell structure. This domain carries the active site as well as an array of subsites each of which interacts with the individual glucose units in the substrate. The specificity of the α-amylases of this family varies and is governed mainly by the position of the catalytic site, the aminoacid composition of the catalytic site, the number of sub-sites and the affinity of each of these sub-sites to the glucose residues in the substrate (MacGregor, 1993, Davies *et al*, 1997). The B domain which is involved in subtrate and Ca^{2+} binding protrudes at the third β strand of the A domain. The C domain follows the A domain and it has been suggested to play a role in enzyme activity (Jespersen *et al*, 1991, van der Maarel *et al*, 2002).

β-Amylase (EC 3.2.1.2) (4-α-D-glucan maltohydrolase, saccharogen amylase, glycogenase, β amylase, β-amylase) is a starch-degrading exo-enzyme belonging to family GH 14. It hydrolyses the (1→4)-α-D-glucosidic linkages in starch, glycogen and related polysaccharides and oligosaccharides to remove successive β-maltose units from the non-reducing ends of the chains. The term 'β' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed. β-amylase cannot pass the α-(1-6) linkages in starch and hence the end-products of β-amylase activity are maltose and β-limit dextrins (Hanes, 1937). The catalytic domain of B-amylases is also a $(\beta/\alpha)_8$ barrell structure.

Glucoamylase (EC 3.2.1.3) (γ-Amylase, Glucan 1,4-α-glucosidase, amyloglucosidase, Exo-1,4-α-glucosidase, lysosomal α-glucosidase, 1,4-α-D-glucan glucohydrolase) is a starch degrading enzyme belonging to family GH 15. It cleaves the

α (1-6) glycosidic linkages, as well as the last α (1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose. Glucoamylases are inverting exo-acting starch hydrolases releasing beta-glucose from the non-reducing ends of starch and related substrates. The catalytic domain of glucoamylases consists of a twisted $(\alpha/\alpha)_6$ barrel with a central funnel-shaped active site connected to a starch-binding domain. They especially hydrolyse α-(1-4) glycosidic bonds but also α-(1-6) glycosidic bonds to a limited extent. (Pazur *et al*, 1960; Sauer *et al*, 2000; Norouzian *et al*, 2006).

II. Alkaline α-amylases from microorganisms

α-amylases are universally distributed throughout the animal, plant and microbial kingdoms (Windish and Mhatre, 1965; Fogarty and Griffin, 1975 and Mantsala, 1989; Lonsane and Ramesh, 1990). However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey, 2000). The major advantage of using microorganisms for the bulk production of amylases is that the microbes are easy to manipulate and enzymes of desired characteristics can be engineered for the economical bulk production (Lonsane and Ramesh, 1990). α-amylases from most bacteria and fungi have pH optima in the acidic to neutral range (Pandey,2000). However, studies on alkalophilic microorganisms have resulted in the discovery of extracellular enzymes that are characterized by high pH optima for activity and stability. Although several extracellular enzymes from alkalophilic microorganisms have been described, there are very few reports on alkaline α-amylases. Alkaline α-amylases have been reported mainly from the genus Bacillus (Horikoshi *et al*, 1982; Horikoshi and Akiba, 1982) with a few reports from other genera ie Myxococcus (Kobayashi *et al*, 1992) and Micrococcus (Farez-Vidal *et al*,1995). Horikoshi (1971) first reported an alkaline α-amylase from an alkalophilic *Bacillus* strain A-40-2. This was followed by the second report of an alkaline α-amylase from *Bacillus* sp. strain NRRL B-3881 by Boyer and Ingle (1972). Subsequently, a number of workers have reported amylases which have pH optima in the alkaline range (Table 1.2). However, as compared to the number of amylases which have pH optima in the acidic or neutral range, the number of alkaline amylases is limited.

An extremely alkalophilic α-amylase with pH optima of 11-12 has been reported from *Bacillus sp*. GM8901 (Kim, 1995). In some cases pH optima was dependent on calcium as in the case of *B. stearothermophilus* (Pfueller, 1969). α-amylases

are generally stable over a wide range of pH from 4 to 11 (Saito *et al*, 1973; Fogarty and Kelly, 1979; Khoo, 1994; Hamilton, 1999.). However, α-amylases with stability in a narrow range have also been reported (Robyt and Ackerman, 1971; Krishnan and Chandra, 1983; Coronado, 2000). The enzyme showed no inhibition by a surfactant or a bleaching reagent. Khan *et al* (2011) also reported a highly alkaline amylase producing *Bacillus megaterium* from enriched soil. *Bacillus* PN5 was isolated from soil which produced a highly thermostable alkaline amylase (Saxena *et al*, 2007). This alkaline amylase was active at pH 9.0 and 90°C suggesting possible application in starch saccharification and detergent formulation.

Alkaline and thermostable α-amylases from *Bacillus halodurans* were isolated and identified as α-amylase I and II. The molecular masses of these α-amylases are 105 kDa and 75 kDa respectively with maximal activities at 50-60°C at pH 10-11 and 42 and 38% relative activities at 30°C. There was no effect of surfactant or bleaching reagents on the enzyme activity. The gene encoding α-amylase I named *amy1* was cloned (Murakami *et al*, 2008). An alkalophilic *Bacillus sp* GM8901 strain grows at pH 10.5 and 50°C. It also produces 5 amylases ie amyI, amyII, amyIII, amyIV and amyV. Amy l has an extremely high optimal pH of 11.0 to 12.0 and SDS PAGE revealed its molecular mass of about 97,000. It has a broad pH range of 6.0 to 13.0 and an optimal temperature of 60°C and was stable up to 50°C (Kim *et al*, 1995). A thermostable amylase produced by *Bacillus stearothermophilus A004* has an optimal temperature of 80°C and extremely broad pH range of 6.0-12.0. It also produces G2, G3, G4 and higher oligosaccharides (Bezbaruah *et al*, 1994).

Table 1.2 : Microorganisms producing alkaline α-amylase

Organisms (sp) ***Alkalophilic / Alkalotolerant***	**Optimu pH**	**Optimum Temp**	**Molecular mass (daltons)**	**Reference**
Bacillus sp. strain A-40-2 (ATCC 21592)	NA	NA	NA	Horikoshi, 1971
Bacillus sp. NRRL B 3881	9.2	50ºC	NA	Boyer and Ingle, 1972
Bacillus licheniformis and	9.5	NA	NA	Medda and Chandra ,1980

Bacillus coagulans				
Bacillus circulans G-6	8.0	60ºC	76,000	Takasaki, 1982
Bacillus licheniformis (CUMC 305)	7.0-9.0	NA	28,000	Kindle (1983), Krish-nan and Chandra (1983)
Bacillus sp. H-167-I *Bacillus sp.* H-167-I *Bacillus sp.* H-167-I	10.5 10.5 10.5	60ºC 60ºC 60ºC	73 000 59 000 80 000	Hayashi *et al*, 1988 a and b
Bacillus sp GM8901	10.5	50-60ºC	97,400 14,400	Shin *et al*,1991
Alkalophilic *bacterium No* 10-1	10.0	50 ºC	NA	Tian *et al*,1991
Natroncoccus Ah36 *(halophilic alkaline amy-lase)*	8-10	55-65°C	70,000 80,000	Kobayashi *et al*, 1992
Bacillus stearothermo-philus A004	6- 12	85 ºC	NA	Bezbaruah *et al*, 1994
Myxococcus coralloides	8.0	NA	NA	Farez-Vidal *et al*, 1995
Bacillus megaterium	9.0-10.0	40ºC	NA	Shiru *et al*, 1994
Bacillus sp. GM8901 Amy I	11 – 12	60°C	97,000	Kim *et al*,1995
Bacillus sp. strain	NA	NA	NA	McTigue *et al*, 1995,

IMD370				Kelly *et al*, 1995.
Bacillus sp. strain NCIB 11203	10.0	NA	NA	McTigue *et al*, 1995
Bacillus halodurans A-59 (ATCC 2159)	NA	NA	NA	McTigue *et al*, 1995
Bacillus megaterium B 9545	10.0	30 ºC	NA	Yan *et al*, (1996)
Halobacterium sp. H-371	9.0	65°C	NA	Zhan *et al*,1996
Thermophillic and alka-lophilic *Bacillus sp.* strain *TS*-23	7.0	70ºC	150,000 42,000	Lin *et al*,1998
Bacillus sp. KSM 1378	8.0	NA	55,391	Igarashi *et al*, 1998
Bacillus sp.	8.0	NA	NA	Ikawa *et al*,1998
Bacillus clausii BT-21	9.5	NA	NA	Duedahl-Olesen *et al*, 2000
Bacillus strain KSM-K2	9.0-10.0	NA	NA	Hiroshi *et al*, 2000
Bacillus KSM 9644	9	55-60 ºC	106,000	Kitayama *et al*, 2000
Ascaris suum (muscle α-amylase)	8.2	NA	NA	Zoltowska, 2001
Bacillus sp.	8.0	NA	55,331	Tull *et al*,2001
Bacillus isolate KSM-K38	8.0 - 9.5	55-60°C	55,000	Hagihara *et al*, 2001

Bacillus amyloliquefa-ciens	10.0	NA	NA	Bessler *et al*, 2003
Thermophilic *Bacillus sp* ANT-6	10.5	80ºC	NA	Burhan *et al*, 2003
Bacillus licheniformis TCRDC-B13	8.0	NA	NA	Bajpai and Bajpai 2004
Bacillus sp. 707	8.8	45ºC	NA	Kanai *et al*, 2004
Bacillus halodurans LBK 34	10.5 – 11.5	60°C	1,07,200	Hashim *et al*,2005
Bacillus sp. AB04	8.0	45ºC	NA	Behal *et al*, 2006
Bacillus halodurans amylase I	10.0	50ºC - 60ºC	105,000	Murakami *et al*, 2007
Bacillus halodurans amylase II	11.0	50ºC - 60ºC	75,000	
Bacillus megaterium	10.0	32ºC - 50ºC	63,000	Khan *et al*, 2011
Bacillus species PN5	10.0	90ºC	NA	Saxena *et al*, 2007
Bacillus strain HUTBS71	5.2-10.0	80ºC - 115ºC	58,800	Al-Quadan Farouk *et al*, 2009
Bacillus subtilis	9.0	50ºC	56,000	Yang *et al*, 2011

***NA- Not available (applicable for all tables wherever NA is used)**

Yang *et al* (2011) reported the cloning of the alkaline α-amylase gene from *Bacillus alcalophilus* JN21 (CCTCC NO.M 2011229) and its expression in *Bacillus subtilis* strain WB600 with vector pMA5. This recombinant alkaline α-amylase showed stability in the pH range 7.0 – 11.0 and temperature below 40°C. This first report about heterologous expression of alkaline α-amylase in *Bacillus subtilis* suggested its possibility in achieving the growing industrial production demand of alkaline α-amylase with its recombinant *Bacillus subtilis* (Yang *et al*, 2011). Some of the properties of the recombinant alkaline amylases have been given in **Table 1.3.**

Table 1.3: Properties of recombinant alkaline amylases

Organism	Optimum pH and temp	Molecular mass	Host	Properties	Reference
Natronococcus sp	9.0, 50°C	55,696	*Haloflex volcanii*	Haloalkalo-philic	Kobayashi *et al*, 1994
Bacillus sp TS-23	7.5-9.4, 65°C	NA	NA	Produces G2/G3/high er oligosac-charides	Lin *et al*, 1994
Bacillus KSM 1378	8.0-8.5, 55°C	53,000	*E.coli HB101*	Produces G3/G5/G6/ G2	Igarashi *et al*, 1998
Bacillus KSM 1378	8.5 40°C	50,000	*B.subtilis*	Liquefying amylasae	Ikawa *et al*, 1998
Pseudomonas spKFCC10818	*8.0*, 40°C	55,696	*E.coli*	Produces G2/G3	Kang *et al*, 2001
B.halodurans amyI,amyII	10.0-11.0, 50-60°C	105,000 75,000	*E.coli*	thermotoler-ant G6 froming	Murakami *et al*, 2007
B.alcalophilus	9.0, 50°C	NA	*B.subtilis*	Used in tex-tile indus-tries and in detergents, laundary	Yang *et al*, 2011

The alkaline α-amylase gene from *Bacillus sp* KSM-1378 was cloned in *E.coli* and *B.subtilis* (Igarashi *et al*, 1998; Ikawa *et al*, 1998). Similarly alkaline α-amylase gene from *Pseudomonas sp KFCC 1018*, *Natronococcus sp*, *Bacillus sp TS-23*, *B.halodurans*, *B.alcalophilus* were cloned. (Kang *et al*,2001; Kobayashi *et al*, 1994; Lin *et al*, 1994; Murakami *et al*, 2007; Yang *et al*, 2011.)

III. Oligosaccharide producing amylases

A number of α-amylases from a wide variety of microbes have been isolated, purified and characterised and have been extensively covered in many reviews (Gupta *et al*, 2003; Pandey *et al*, 2000).

In recent years, there has been an increasing interest in the use of malto-oligosaccharides as biopreservatives (Crittenden and Playne, 1996; Barreteau *et al*, 2006). Traditionally, malto-oligosaccharides which are the products of starch liquefaction have a degree of polymerization (DP) between 2 and 10 (G2-G10). Since these oligosaccharides are natural components of foods such as fruit, vegetables, milk and honey, they are increasingly being favoured in lieu of chemical additives which are becoming less welcome by the consumers (Barreteau *et al*, 2006; Mussatto and Mancilha, 2007.) Amongst the malto-oligosaccharides, G3 (maltotriose), G4 (maltotetraose), G5 (maltopentaose) and G6 (maltohexaose) have attracted attention because they not only provide useful modifications to flavour and physicochemical characteristics of the food, but also have properties that are beneficial to human health (Crittenden and Playne, 1996; Barreteau *et al*, 2006). Major uses of maltooligosaccharides are in beverages, infant milk powders, confectionery, bakery products, yoghurts and dairy desserts.

The chemical manufacture of maltooligosaccharides larger than maltotriose has been very difficult. However, the discovery of microbial enzymes that specifically act on starch substrate to produce specific malto-oligosaccharides has made it possible to produce syrups containing various malto-oligosaccharides (Okada and Nakakuki, 1992). The price of pure maltooligosaccharides is extremely high due to difficulties in obtaining them in high yield and in a pure state. Thus, it has become more important to develop efficient synthesis routes, easy to scale-up, so as to obtain such useful oligosaccharides. The broadening of the application of such enzymatic synthesis routes requires the identification and characterization of new glycosidases and glycosyltransferases through classical microbial screening as well as by site-directed mutagenesis of enzymes of known structure. The market for these oligosaccharides in Japan alone was projected to be more than 20 billion yen/year (Nakakuki, 2003).

Not all amylases produce specific malto-oligosaccharides. Specific malto-oligosaccharide (G3, G4, G5, G6 and G7) producing α-amylases have been mainly identified in bacteria. Tables 1.4, 1.5, 1.6, 1.7 and 1.8 summarize the list of organisms producing G3, G4, G5, G6 and G7 producing amylases.

Table 1.4: Maltotriose (G3) forming microorganisms

Organism	**Optimum pH and Temperature**	**pI**	**Molecular Mass**	**Reference**
Streptomyces sp.	Neutrophilic	NA	NA	Wako *et al*, 1979
Bacillus subtilis	pH 8.0, 30^0C	NA	NA	Takasaki, 1985
Microbacterium imperiale	pH 7.0, 30^0C	NA	NA	Takasaki *et al*, 1991
Natronococcus sp. Strain Ah36	pH 8.7, 55^oC	NA	74,000	Kobayashi *et al*, 1992
Chloroflexus aurantiacus	pH 7.5 ,71°C	6.24	210,000	Ratanakhanokchai *et al*,, 1992
Streptococcus bovis 148 (endo-amylase)	pH 6.5, 40^0C	NA	57,000	Satoh *et al*,1993,1997
Thermobifida fusca	pH 7.0, 60^0C	4.55	60,000	Chao-Hsun and Wen-Hsiung 2004
L. amylophilus GV6	pH 6.5, 37 ^{o}C	NA	NA	Vishnu *et al*, 2006

Table 1.5: Maltotetraose (G4) forming microorganisms

Organism	**Optimum pH and temp**	**pI**	**Molecular Mass (daltons)**	**Reference**
Pseudomonas stutzeri ***(exo-amylase)***	pH 8, 45 ^{o}C (two forms)	**F_1**-5.6 **F_2**-5.3	55,000	Sakano *et al*, 1983; Robyt and Ackerman, 1971

B.circulans	pH7.0, 50°C	NA	45,000	Takasaki, 1983
Pseudomonas stutzeri MO-19	pH 7.0, 50°C (two forms)	**(G_4-1)**-5.0 **(G_4-2)**-4.8	**(G_4-1)**-57,000 **(G_4-2)**-46,000	Nakada *et al*, 1990
Bacillus circulans spMG- 4 ***(exo and endo)***	pH 7.5, 50°C	NA	10,000	Takasaki *et al*, 1991
Chloroflexus Auranticus	pH 7.5, 71°C	6.24	210,000	Ratanakhanokchai *et al*, 1992
Pseudomonas sp. IMD 353 ***(endo-amylase)***	pH 7.0, 50°C	5.0	63,0000	Fogarty *et al*, 1994 a and b
Streptomyces sp KSM-35	pH 6.0, 50 – 60 °C	4.3	55,000	Cha *et al*, 1994
Pseudomonas sp. MS300	pH 6.8, 40°C pH 6.8, 40°C	5.0 4.7	55,000 55,000	Kobayashi *et al*, 1998
Bacillus sp IMD 370 *(raw starch digesting amylase)*	pH 8.0 (raw starch) pH 10.0 (soluble)	NA	NA	Kelly *et al*, 1995
Bacillus sp GM 8901	pH 11.0-12.0, 60°C	NA	97,000	Kim *et al*, 1995
Bacillus subtilis KSM 103	pH 5-7, 65°C-70°C	NA	53,000	Nagarajan *et al*, 2006

Table 1.6: Maltopentaose (G5) forming microorganisms

Organism	Optimum pH and temperature	Molecular Mass (daltons)	Reference
Bacillus licheniformis	pH 7.2,50°C	NA	Saito, 1973
Bacillus licheniformis	NA	NA	Pankratz, 1977
Streptomyces myxogenes SF-1130	NA	NA	Iwamatsu *et al*, 1979.
Bacillus circulans	pH 7.0, 50°C	45,000	Takasaki, 1983
Bacillus cereus NY-14	NA	NA	Taniguchi *et al*,1983
Pseudomonas sp (KO-8940)	pH 8.0, 45°C	NA	Okemoto *et al*, (1986)
strain 163-26	NA	*(AmylaseA-60)-60kDa (AmylaseA-180)-180kDa*	Schmid *et al*, 1993
Pseudomonas spp	NA	NA	Shida *et al*, 1992
Bacillus licheniformis	pH 8.0-8.5, 55°C	53,000	Igarashi *et al*, 1998
Bacillus strain GM8901	pH 11.0-12.0	97,000	Kim *et al*, 1995
Bacillus circulans	pH 4.5, 48°C	NA	Dey *et al*, 2003

Table 1.7 : Maltohexaose (G6) forming microorganisms

Name of the Organism	Optimum pH and Temperature	pI	Molecular mass	Reference
Aerobacter aerogenes	pH 7.0, 45°C	NA	54,000	Kainuma *et al*, 1975
Streptomyces myxogenes SF-1130	NA	NA	NA	Iwamatsu *et al*, 1979
Bacillus circulans	pH 8.5, 60°C	NA	76,000	Takasaki. 1982

G-6				
Bacillus circulans F-2	pH 7.0, 30°C	NA	93,000	Taniguchi *et al*, 1982
Bacillus sp H-167	pH 9.4, 37°C	NA	**H-I-1-** 73,000 **H-I-2-** 59,000 **H-I-1-** 80,000	Hayashi *et al*, 1988a and b
Bacillus sp 707	pH 8.8, 45°C	NA	NA	Tsukamoto *et al*, 1988
Bacillus caldovelox	Acidophilic	NA	NA	Fogarty *et al*, 1991
Bacillus strain GM8901	pH 11.0-12.0 60°C	NA	97,000	Kim *et al*, 1995
Alcaligenes latus FERM BP-4578	pH5.0, 70°C	7.6	43,000	Nakano *et al*,1998 US 5,846,808
Bacillus licheniformis	pH 8.0-8.5 55°C	NA	53,000	Igarashi *et al*, 1998
Bacillus clausii BT-21 ***(exo-amylase)***	alkalotolerant pH 10, 45°C	NA	101,000	Duedahl-Olesen *et al*, 2000
Bacillus subtilis US116	NA	NA	NA	Messaoud *et al*, 2004
Bacillus halodurans LBK 34	pH 10, 37°C	4.41	107200	Hashim *et al*, 2005
B.stearothermophilus US100	pH 5.6, 80°C	NA	NA	Ben Ali *et al*, 2 006

Table1.8: Maltoheptaose (G7) forming microorganisms

Organism	Optimum pH and temperature	Molecular Mass (daltons)	Reference
Alcaligenes latus FERM BP-4578	pH 5.0, 70°C	43,000	Nakano *et al*, 1998 US 5,846,808
Bacillus isolate KSM-K38	pH 8.0-9.5 55-60°C	55,000	Hagihara *et al*, 2001
Alcaligenes sp	pH4.0-5.0, 70°C	NA	Nakano *et al*, 2001 US 6,242,22481
Pyrococcus furiosus	pH 4.5, 90°C	76,084	Yang *et al*, 2004
Bacillus subtilis	pH 6.0, 37°C	NA	Messaoud *et al*, 2008

Cloning and expression of genes encoding malto-oligosaccharide forming α-amylases

Table 1.9 summarises information on the various genes encoding maltooligosaccharides forming α-amylases from various microbial sources.

Table 1.9: Cloning of the genes encoding maltooligosaccharides forming α-amylases from various microbial sources

Organism	Expression host	Gene size (bp)	Leader sequence	Properties	Reference
B.amyloliquefaciens	*B.subitilis*	1542 (preamylase) 1449 (secreted α-amy)	31	NA	Takkinen *et al*, 1982
Pseudomonas stutuzeri MO-19	*E.coli*	1641	21	Produces G4	Fujita *et al*, 1989
Pseudomonas saccharophila	*E.coli*	1653	21	maltoteraohydrolase production	Zhou *et al*, 1989
Natronococcus sp. strain Ah-36	*Haloflex volcanii*	1512	43	Produces G3	Kobayashi *et al*, 1994

Cryptococcus species	*Saccharomyces cerevisiae*	1833	20	Produces G1/G2/G3/G4	Iefuji *et al*, 1996
Pyrococcus furiosus	*E.coli and B.subtilis*	1383	25	Industrial application due to high thermostability ,no metal requirement, substrate specificity, produces small linear and branched oligosaccharides	Jorgensen *et al*, 1997
Streptococcus bovis	*E.coli*	1452	Does not contain	Produces 2 intracellular α-amylase forming G2/G3/G6	Satoh *et al*, 1997
B.subtilis SUH4-2 *Streptomyces albus KSM-35*	*E.coli and B.subtilis*	NA	NA	Produces G3/G6	Min *et al*, 1998
Bacillus isolate KSM 1378	*E.coli*	1548	31	Novel semi-alkaline liquefying amylase froming G3/G5/G6/G2	Igarashi *et al*, 1998
Bacillus isolate KSM	*B.subtilis*	1548	31	Novel	Ikawa *et al*,

1378				semi-alkaline liquefying amylase froming G3/G5/G6/G2	1998
Bacillus sp. US100	*E.coli*	NA	NA	Produces thermoactive α-amylase froming G6	Ben Ali *et al*, 1999
Pseudomonas stutzeri	*E.coli TPS618*	1578	21	DNA encoding a polypeptide possessing maltotetraose forming amylase activity.	US Patent 5958749 Kubota *et al*, 1999
Klebsiella pneumoniae	*E.coli*	2031	NA	Produces G6 amylase	Momma, 2000
Pseudomonas sp KFCC10818	*E.coli*	1356	NA	Produces G2/G3	Kang *et al*, 2001
Bacillus halodurans MS-2-5	*E.coli*	2874 (overlapped 4 DNA fragments)	NA	Produces G1/G2/G3/G4/G6	Murakami *et al*, 2007
Sclerotinia sclerotiorum		1500	21	Produces G3	Abdelmalek, I *et al*, 2008
Brachybacterium sp strain LB25	*E.coli*	1722	NA	Produces G4	Doukyu *et al*, 2008
Thermobifida fusca NTU22	*P.patoris*	NA	NA	Produces G3	Yang and Liu, 2011

Hosts commonly used for cloning

Yang *et al* (2010) used *P. pastoris* as an expression host to clone the α-amylase gene from *Thermobifida fusca.* There was a rapid production of the extracellular amylase obtained which lead to an increase in biomass accumulation of *P. pastoris* transformant. It was found that this system is more suitable for large scale production. This was mainly due to the elimination of the hazard and cost associated with the storage and delivery of large volumes of methanol (Goodrick, 2001). These features of the expression system may significantly contribute to the development of cost-effective methods suitable for large-scale production of heterologous recombinant proteins. Yang *et al* (2011) used *B.subtilis* as an expression host. Though there is an increasing interest in alkaline amylases, the yield of α-amylase is very low. The overproduction can be achieved with a combination of optimized process and genetic engineering. (Yang *et al*, 2011). *B.subtilis* is known as an efficient expression host for production and secretion of proteins, and is one of the expression hosts used for the industrial production of enzymes. It is used in areas like production of interferon (Palva, 1983), insulin (Olmos-Soto *et al*, 2003) pathogenic antigens (Airaksinen *et al*, 2003) and toxins (Taira *et al*, 1989) and enzymes of great industrial use like proteases (Ho *et al*, 2003), α-amylases (Huang *et al*, 2004) and lipases (Ho *et al*, 2003). The most significant benefit of *B.subtilis* when compared with the other expression host production systems are (i) high cell density growth and (ii) secretion of the synthesized protein in the cultivation medium. This actually eases the process of isolation and purification of the protein during the downstream processing (Bron *et al*,, 1998; Liu *et al*, 2004). *B.subtilis* is also used for the production of homologous or heterologous proteins. Here, the desired protein is fused to the α-amylase promoter and leader peptide leading to an efficeint secretion of proteins in the culture medium (Airaksinen *et al*, 2003**;** Ho *et al*, 2003.).

A eukaryotic host is generally preferred for high level expression of many eukaryotic proteins for structural analysis. This is due to the insolublility of proteins or lack of essential post-translational modifications when expressed in *E. coli*. The eukaryotic expression host system *Saccharomyces cerevisiae* is chosen as it possesses several advantageous features of a good expression host: (i) easy to culture and inexpensive (ii) has excellent recombinant DNA tools. Some more properties of the eukaryotic expression system *S.cerevisiae* which makes them desirable are as follows (a) expression in yeast significantly improves the solubility of proteins that are

expressed but these proteins are insoluble in *E. coli*. (b) Small scale purifications have shown that the yield and purity of heterologous proteins expressed in yeast is sufficient for structural analysis. (c) By altering the codon usage, the yield of protein expression can be improved. From the above mentioned features of *S.cerevisiae* it is evident that it provides high level expression and purification of heterologous proteins and there is a possibility of improving the expression of proteins which are poorly soluble in *E. coli* (Quartley *et al*, 2009).

IV. α-*Amylases from Streptomyces*

Streptomyces are gram positive mycelial soil bacteria with high GC content. *Streptomyces species* are very well known to produce several extra-cellular enzymes which are used for decomposing plant material. The ability to produce several hydrolytic enzymes enables *Streptomyces* to grow on polymeric substrates. (Gilbert *et al*, 1995). The hydrolytic enzymes such as proteases, nucleases, lipases, amylases secreted by the *Streptomyces* are used to hydrolyze/degrade different types of complex polysaccharides present in organic debris from which these organisms derive their nutrition (Williams *et al*, 1983). It is due to this ability to utilize simple and complex molecules that they are termed as heterotrophic feeders. They are also known to produce many secondary metabolites and proteins which makes them a popular choice in industries.

The few α-amylases reported from the genus *Streptomyces* have acidic or neutral pH optimum **(Table 1.10).**

Table 1.10: Streptomyces species producing alpha-amylases

Species	Properties	Mol. Wt (kDa)	Optimum pH and temp	Reference
S. albus	NA	NA	5.7	Hyslop and Sleeper, 1964.
S. praecox	NA	NA	6.0	Takaya *et al*, 1979; Suganuma *et al*, 1980.

S. hygroscopicus SF 1084	NA	NA	5 – 6 50˚C-55˚C	Hidaka *et al*, 1974, 1978; Hidaka and Adachi, 1980.
S. limosus	NA	NA	7.0	Fairbarn *et al*, 1986.
Streptomyces sp. KSM-9	NA	NA	9.0	Nakai *et al*, 1986 (PATENTED)
S. rimosus	Partially stabilized by Ca^{2+}, forms G3/G4/higher oliosaccharides	43	5 – 6	Vukelic *et al*, 1992.
Streptomyces sp. KSM-35	Produces G2,G3,G4	NA	6.0	Cha *et al*,1994
S.rimosus TM-55	NA	NA	6 – 7	Cheng and Yang, 1995;Yang and Cheng, 1996; Yang and Wang, 1999.
S. thermocyaneoviolaceus IFO14271	NA	NA	6.5	Hang *et al*, 1996
Streptomyces sp. IMD 2679	G2 forming	47.8	5.5	McMahon *et al*, 1997,1999
S. megasporus	Thermostable upto 85˚C with half life 60 min, forms G3/G2	97	6.0, 50˚C-60˚C	Dey and Agarwal, 1999

Streptomyces sp TOTO 9805	NA	NA	10.0	Moriyama *et al*, 2000 (PATENTED)
Streptomyces sp No. 4	Forms G2/G3/G4/dextrin	NA	5.5, A1-50˚C A2-45˚C	Primarini and Ohta, 2000
Streptomyces sp.	NA	NA	6 – 7	Ammour *et al*, 2002
Streptomyces sp E-2248	Forms G2 from gelatinized starch	47	6.0	Kaneko *et al*, 2005
S. aureofaciens	calcium independent, G3/G2 forming	40	4.6 – 5.3, 40˚C	Hostinova *et al*, 2006
S. gulbargiensis	Alkali-thermotolerant, produces G2, G3	55	11.0	Syed *et al*, 2009
Streptomyces sp. D1	application in detergents and pharmceutics	66	9.0 45ºC	Chakraborty *et al*, 2009

To date there are only four reports of alkaline amylases from this genus, two of which are patented. (Nakai *et al*, 1986; Moriyama *et al*, 2000; Chakraborty *et al*, 2009; Syed *et al*, 2009). The alkaline amylase reported by Moriyama *et al* (2000) was found to be optimally active at pH 10.0 and 55ºC. The enzyme had a molecular weight of 77,500 daltons as estimated by SDS-PAGE. The alkaline amylase patented by Nakai *et al* (1986) has optimum temperature and pH of 30^0C and 9.0 respectively.The strain reported by Nakai *et al*, (1986) produces 0.85 IU/ml of amylase.

Some of the important properties of the alkaline amylases produced by alkalophilic *Streptomyces* strains are shown in Table 1.11.

Table1.11: Characteristics of alkaline alpha amylase from Streptomyces

Organisms (sp)	**Optimum pH**	**Optimum Temperature**	**Molecular weight**	**Reference**
Streptomyces sp.KSM-9	9.0	30ºC	NA	Nakai *et al*, 1986 (PATENTED)
Streptomyces TOTO – 9805	10.0	50ºC	77,500	Moriyama *et al*, 2000 (PATENTED)
Streptomyces sp. D1	9.0	45ºC	66,000	Chakraborty *et al*, 2009
S.gulbargensis	8.5–11	45ºC	55,000	Syed *et al*, 2009

In the genus *Streptomyces*, to date there is one report of a neutrophilic strain (Wako *et al*, 1979) and two reports of alkalophilic strains producing maltotriose (Chakraborty *et al*, 2009; Syed *et al*, 2009). There is only one report of an acidophilic strain producing maltotetraose (Cha *et al*, 1994). *S. gulbargensis* also produces maltotetraose in addition to maltotriose (Syed *et al*, 2009).

α-Amylases from different *Streptomyces* species have been cloned and expressed in different hosts (Table 1.12).(McKillop *et al*, 1986; Hoshiko *et al*, 1987; Long *et al*, 1987; Virolle and Bibb, 1988; Bahri and Ward, 1993; Tsao *et al*, 1993; Vigal *et al*, 1991, 1994;Chen *et al*, 1995; Kim *et al*, 1996; Yin *et al*, 1997; Mellouli *et al*,1998).

Table1.12: Genes encoding α-amylases from Streptomyces species that have cloned and expressed in different hosts.

Organism	Expression host	Gene size (bp)	Leader sequence (amino acids residues)	Reference
Streptomyces hygroscopicus	*S.lividans 66*	NA	NA	McKillop *et al*, 1986
Streptomyces hygroscopicus	*E.coli*	1434	30	Hoshiko *et al*, 1987
Streptomyces limosus	*Streptomyces lividans*	1690	28	Long *et al*, 1987
Streptomyces venezuelae ATCC15068	*Streptomyces lividans*	1722	NA	Virolle and Bibb,1988
Streptomyces griseus IMRU 3570	*Strptomyces lividans*	NA	28	Garcia Gonalez *et al*, 1991
Streptomyces thermoviolaceus CUB74	*E.coli and S.lividans*	1380	NA	Bahri and Ward, 1993
Streptomyces lividans TK24	*S.lividans*	2757	33	Tsao *et al*, 1993
Streptomyces griseus	*E.coli*	1698	28	Vigal *et al*, 1994
Streptomyces lividans TK24	*Streptomyces lividans TK24*	2978	NA	Yin *et al*, 1997
Streptomyces sp. TOI	*E.coli*	1359	NA	Mellouli *et al*, 1998
Streptomyces albus KSM-35	*E.coli and B.subtilis*	NA	NA	Min *et al*, 1998

Streptomyces are saprophytic soil bacteria producing several kinds of secondary metabolites, including most of the known antibiotics. Genes encoding a variety of extracellular enzymes from *Streptomyces* species have been cloned and characterised. These include endoglycosidase H (Robbins, 1981), agarase (Kendhall and Cullem, 1984), xylanase (Iwasaki, 1987) and amylases (McKillop,1986; Hoshiko,1987; Long *et al*, 1987; Virolle and Bibb, 1988; Vigal,1991; Tsao,1993; Chen, 1995). Since protein secretion often coincides temporally with secondary metabolism (Chater,1984), the nucleotide sequence of the control regions of these genes may provide insights into the regulation of protein secretion and secondary metabolism of *Streptomyces* sp. *S.hygroscopicus* SF-1084 produces an extracellular α-amylase which is used industrially to hydrolyze starch to maltose. Study on cloning provides insights on regulation of the gene and the properties of its products. Such studies have helped to maximize the efficiency of hydrolysis and to better understand the control of gene expression in *Streptomyces* sp (Hoshiko *et al*, 1987). The 30-residue leader sequence showed similarities to those found in other procaryotes. The amino acid sequence of the amylase structural gene indicates several conserved features. Three regions which are conserved in other α-amylases were also found in *Streptomyces* α-amylase. The streptomycete gene is unique in that it has a tryptophan residue (Trp291) in the third conserved region. All other α-amylases studied possess histidine in this position of the third conserved region (Matsuura, 1984; Ihara, 1985; Nakajima, 1986).

Isolation and sequencing of the *amy* gene coding the α-amylase (E.C 3.2.1.1) from *S.hygroscopicus* has been done by Hidaka *et al*, (1974). The *amy* nucleotide sequence indicated that it coded for a protein of 52 kDa (478 amino acids). The amylase gene was cloned into cosmid vector pHC79. Similarity was seen between the 30 residue leader sequences of this amylase gene with other prokaryotes. Amino acid sequencing of the *amy* structural gene indicates several conserved features and three regions which are conserved in other α-amylases (Matsuura, 1984; Ihara,1985; Nakajima,1985). Some of the cloned genes are convenient for the study of gene expression and their regulation in *Streptomycetes*, and all have potential application for the construction of expression and secretion vectors for the production of heterologous proteins in this industrially familiar genus. Substantial nucleotide and amino acid sequence data on these genes and proteins are now made available. Previously a comparison of the amino acid sequences of 11 different α-amylases was done by Nakajima *et al*,(1986). While considerable amino acid sequence identity could be

detected between six mammalian a-amylases (ranging from 80 to 90%), minimal (<10%) identity was detected between these enzymes and α-amylases from a wide range of other species, including several bacteria, *Aspergillus oryzae*, and barley. In a comparison which involves α-amylases from a wider variety of genera, it was observed that there is a considerable amino acid sequence identity between the α-amylases of *S. limosus* and those of mammalian and invertebrate origin. There is also a considerable degree of amino acid sequence identity (36%) between the carboxy-terminal portion of the *S.limosus* α - amylase and glucoamylase of *A.niger*.

The gene encoding maltotetraose and maltotriose (designated amylase IV) from *S.albus* KSM-35 (Cha, 1994) was cloned (Lee, 1996a) and expressed in *B.subtilis* with the help of a α-amylase based secretion vector by using the method of Sadie and Kada (1983) with with minor modification (Lee, 1996b).

V.Studies on structure-function relationships in α-amylases

The properties of enzymes can be correlated with the amino acid residues present in their active site and also their architechture. X-ray crystallography, homology modeling, chemical modification, fluorescence and circular dichroism have provided valuable information on the structure-function relationship. The use of these techniques in understanding the properties of microbial α-amylases is discussed below.

(i) X-ray crystallography studies

The study of the crystal structure along with sequence information of homolgues and homology modeling studies has helped in deciphering structure-function relationship and in identifying beneficial substitutions that could help to design enzymes with improved properties to suit various industrial needs (Nielsen and Borchert, 2000). The α-amylases have a common three-dimensional structure comprising three domains ie A, B and C domains. Domain A is a $(\beta/\alpha)_8$ barrell structure which is the catalytic core. Domain B is formed due to an excursion between barrel strand β-3 and helix α-3, while domain C which is frequently an eight stranded β-sheet, which lies at the C terminal extremity of the barrel (Brzozowski, 2000). Structural studies have helped to determine the architecture of the overall polypeptide and active site. Some family members may contain extra domains. For eg; the *B.stearothermophilus* α-amylase consists of five domain structures with some of the additional domains involved in binding to granular starch. (Strokopytov, 1995, 1996; Dauter, 1999). Solving the crystal structure could aid in rational designing of inhibitory agents that provide therapeutic opportunities in a number of areas, including the control of

diabetes, hyperlipoproteinemia and possibly obesity. (Truscheit *et al*, 1981; Nishide *et al*, 1986; Clissord and Edwards,1988). The α-amylases from micro-organisms whose three-dimensional structures have been solved by X-ray crystallography are shown in Table 1.13.

Table 1.13: Microorganisms whose alpha-amylase structure has been solved by X-ray crystallography

Organism	Reference
Bacillus sp 707	Kanai *et al*, 2004
B. stearothermophilus	Suvd *et al*,2001; Ben Ali *et al*, 2006.
B. licheniformis	Joyet *et al*,1992; Conrad *et al*, 1995; Hwang *et al*,1997; Brzozowski *et al*, 2000; Machius *et al*, 1995, 1998, 2003; Declerck *et al*, 1995, 1997, 2000, 2002.
B. subtilis	Kagawa *et al*, 2003; Nonaka *et al*, 2003.
Bacillus sp. Strain KSM-K38	Hagihara *et al*, 2001b.
B. amyloliquefaciens	Welker and Campbell,1967. Brzozowski *et al*, 2000.
Bacillus sp KSM-1378	Ikawa *et al*, 1998; Shirai *et al*, 2007.
* *Thermoactinomyces Vulgaris*	Kamitori *et al*, 1999, 2002; Mizuno *et al*, 2004.
Alteromonas haloplanetis	Aghajari *et al*, 1998
Aspergillus sp	Matsuura *et al*, 1984; Boel *et al*, 1990; Qian, 1994; Vujicic-Zagar and Dijkstra, 2006.
Klebsiella pneumonia	Momma *et al*, 2004
Halothermothrix orenii	Li *et al*, 2002; Tan *et al*, 2003
Pseudomonas stutzeri	Morishita *et al*,1997; Mezaki *et al*, 2001

A number of properties of α-amylases that been deciphered from the crystal structure are briefly discussed below:

Alkaline adaptation : Shirai *et al*, (2007) have used the crystal structure of the alkaline α–amylase (Amy K) from *Bacillus sp*. KSM-1378 and the ASET method (ancestral

sequence Evolutionary Trace Method), wherein comparisons were made between the alkaline and non-alkaline enzyme structures from proteases, cellulases and phosphoserine aminotransferases, to explain the mechanism of alkaline adaptation of these enzymes. Their study revealed that a decrease in the Lys residues resulted in a decrease in Lys-Asp/Glu ion pairs and was compensated by acquisition of Arg-Asp-Glu. Since Arg have a higher pKa than Lys, this ion-pair remodelling resulted in a shift of the optimum pH of the enzymes to the alkaline side. Shaw *et al* (1999), have identified determinants that improved the performance of *B.liceniformis* alpha-amylase at pH<6.

Thermostability: Declerck *et al* (2000) investigated the relationship between thermostability and structure using the mutation analysis of BLA, and found that some of the amino acid changes contribute to the differences in thermostability between BLA (*Bacillus licheniformis amylase)*, BAA (*Bacillus amyloliquefaciens amylase)* and BSTA (*Bacillus stearothermophilus amylase*). In BLA, replacement of Ala 269 by Lys caused a reduction in the thermostability. However, the corresponding residue, Lys 272, in BSTA may not be destabilizing because it forms a salt bridge with Asp l90, which is Ser in BLA and thus cannot form such a salt bridge (Suvd, 2001). Suvd *et al*, (2001) explained the differences in thermostability between the amylases of *B. stearothermophilus* (BSTA), *B. amyloliquefaciens* (BAA) and *B.licheniformis* (BLA) on the basis of the crystal structure. BSTA is a thermophilic enzyme which shows 65% and 66% sequence identity with BLA and BAA respectively. The crystal structures of BSTA was compared with that of BLA with respect to overall topology, hydrogen bonding, metal ion binding, hydrophobic interactions and the accessible area and cavities. In BLA, Asp 204 coordinates the Ca^{2+}. In BSTA, a two aminoacid insertion ie Ile 181- Gly 182 pushes away the spatially contacting region having Asp 207 which corresponds to Asp 204 in BLA. Due to this, a water molecule coordinates the Ca^{2+} in BSTA instead of the side chain of Asp 207. Besides this, BSTA has nine fewer hydrogen bonds and voids are observed in th $(\beta/\alpha)_8$ barrel which decrease the inter-helical compactness and hydrophobic interactions. The percentage of solvent accessible surface area of charged residues is two times larger than that in BSTA. Similar studies for improving the thermostability and half life of the alpha-amylases have been done in *B. stearothermophilus* (Suvd *et al*, 2000, 2001; Ben Ali *et al*, 2006) and *B. licheniformis*

(Joyet *et al*, 1992; Conrad *et al*, 1995; Declerck *et al*, 1995, 1997, 2000, 2002; Machius *et al*, 1998, 2003).

Cold adaptation: Aghajari *et al* (1998) explained the determinants of cold adaptation of the α-amylase from the psychrophilic *Alteromonas haloplanctis* by comparing its structure with the available structures from the wild-type and mesophilic counterparts. Their study revealed that the conformational flexibility of the cold-adapted enzyme allows the enzyme to function efficiently at low temperatures. The factors which contributed to the high flexibility of the psychrophilic α–amylase were (i) reduced number of charged residues which were responsible for the reduced number of hydrogen-bonding thereby allowing increased penetration of the solvent. (ii) the core of the psychrophilic enzyme had reduced number of proline and arginine residues making it less compact and hence more flexible (iii) inter-domain interactions were either weak or were lacking and (iv) increased number of hydrophobic residues increased the exposure of the side chains to the solvent.

Mechanism of chelator resistance and oxidative stability: All known α-amylases contain calcium ions that contribute to stabilization of the structures (Shirai, 1997). Furthermore, all known α -amylases, except for cyclodextrins- and pullulanhydrolyzing α-amylases containing an additional domain, have a common site for the highly conserved calcium ion at the interface between two domains (A and B) to keep the functioning structure (Nielsen and Borchert, 2000). The role of the conserved calcium ion is mainly to retain the structural rigidity of the α-amylase molecules (Larson, 1994; Janecek *et al*, 1997). It is only due to these crystal structure studies that the essential role of **'Ca^{2+}'** can be explained in α-amylases by the observation that its ligands belong to domain A and domain B and that the active site cleft by inducing an ionic bridge between domains A and B. Hence the essential **'Ca^{2+}'** ion appears to stabilize the active site cleft by inducing an ionic bridge between domains A and B.

Nonaka *et al* (2003) determined the crystal structure of AmyK38 from *Bacillus sp*. strain KSM-K38 by the molecular replacement method. This amylase is a calcium-free amylase which resists chelating agents and chemical oxidants. The study of its crystal structure was able to explain why this enzyme retained its activity in presence of chelating agents. The crystal structure showed that the highly conserved calcium ion which is located at the interface between domains A and B in other amylases, is absent

in this amylase. In addition, this amylase does not have other calcium ions. Instead, three Na^+ ions were found of which one is located at the position corresponding to that where the highly conserved Ca^{2+} ion is found in other amylases. Their study suggested that these Na^+ ions are used to retain the structure and function of AmyK38 in place of the Ca^{2+} ions. When the structure of AmyK38 was compared with the *B.licheniformis* amylase BLA, it was found that the Asp 161, Asp 183, Asp 200 and Asp 204 around the Ca-Na-Ca triad containing the highly conserved Ca I in BLA were replaced with Asn 161, Asn 183, Asn 200 and Ser 204 in AmyK38. Asp 430 at the Ca III site in BLA was replaced with Asn 427 at site II in AmyK38. These replacements reduced the negative charges at the cation binding sites as a result of which monovalent ions such as Na^+ bind in the structure in place of a divalent metal ion such as $Ca^{2+.}$ Their study suggested that site directed mutagenesis experiments can be directed to replace negatively charged aminoacids with neutral or positively charged amino acids to engineer amylases which would find application in the detergent industry.

Wide substrate specificity : The mechanism underlying the wide substrate specificity of the α–amylase II (TVA II) of *Thermoactinomyces vulgaris* was determined by Mizuno *et al* (2004) using the crystal structure of TVA II complexed with 4^2- α – panosylpanose which is a pullulan hydrolysate composed of two pannose units. TVA II is an intracellular enzyme which hydrolyses the α -1,4 – glucosidic linkages in starch, pullulan and cyclodextrins and also the α -1,6 – glucosidic linkages of isopanose. The crystal structure of TVA II revealed that it forms a dimeric structure (Ibuka *et al*, 1998; Kamitori *et al*, 1999). Each monomer unit consists of domains A, B and C which is present in all α–amylases. However, unlike other α–amylases, TVA II has a N domain at the N terminal end comprising of 120 amino acids.The N domains of both the monomers along with the A domains form the active cleft of the enzyme.The loop formed by residues 193-218 is located at the end of the active cleft around the non-reducing region and forms a `dam' like bank which gives the active cleft its unique shape. In TVA II, the loop is short which makes the active cleft wide and shallow around the non-reducing region, conferring the wide substrate specificity to this enzyme.

Mechanism of maltooligosaccharides formation: The mechanism of reaction of the usual α-amylases varies from that of the α-amylases producing oligosaccharides. It is assumed that these oligosaccharides producing α-amylases have four, five or six subsites from the active center. This particularly produces oligosaccharides and also protects them from further degradation.

The crystal structure of maltotetraose (G4) forming amylase from *Pseudomonas stutuzeri* has been reported by Yoshioka *et al* (1997) and Mezaki *et al* (2001). Yoshioko *et al* (1997) described two crystal structures of a mutant enzyme whrein the essential catalytic residue Glu 219 was replaced by Gln (E219Q). In order to observe the structure of substrate–bound enzyme the crystal was co-crystallized with maltopentaose. Crystal structure of G6 forming α-amylase from alkaliphilic *Bacillus sp 707* also has been reported by Kanai *et al* (2004).

The crystal structure of the mutant G4-α-amylase complexed with maltotetraose has revealed that G4 bears 4 subsites at the non-reducing side and with several hydrogen bonds at subsite -1 it binds the glucosyl residue (Yoshioka, 1997). It is expected that G6 amylase has at least 6 subsites at the non-reducing end and has a specific interaction of subsite-6 with a sugar residue. Predominant production of a specific oligosaccharide requires controlled and strong interaction between the glucosyl residue and the subsite of the non-reducing end of the oligosaccharide formed. It is suggested that there are structural differences between G6- α-amylase and G4- α-amylase (Kanai *et al*, 2004). Kanai *et al* (2004) have aso commented on the significant role of Trp 140 on maltohexaose production from G6- α-amylase isolated from alkaliphilic *Bacillus sp 707*. This study was based on the activities measured for the mutant G6- α-amylase with replacement of Trp-140 with Leucine (W140L) or Tyrosine (W140Y).

Mechanism of acidostability: The molecular basis of acidostability was studied using sequence information of the α-amylase from *Alicyclobacillus acidocaldarius.* (Schwermann, 1994) aligned the sequence of the α-amylase from *Alicyclobacillus acidocaldarius* ATCC 27009 with its neutrophilic relatives and also with the α-1,4 and α-1,6 glycosidic bond hydrolysing enzymes of known three dimensional structures to investigate the determinants of acid stability. Their study revealed that the acidophilic α-amylase has 30% less charged residues which are replaced by neutral polar residues on the surface of the protein. The proteins that have adapted to the acidic environment have

reduced density of both positive and negative charges on their surface which reduces the electrostatic repulsion at low pH thereby conferring stability in an acidic environment.

(ii) Chemical modification of residues in the active site

The chemical modifications of enzymes provide a means of elucidating enzymatic properties. Chemical modification of proteins is widely used as a tool for studying localization of individual amino acids, their participation in the maintenance of the native conformation and for their stabilization (Torchilin *et al*, 1979; Miland *et al*, 1996). This approach was initially used to identify specific residues at active sites involved in substrate binding or chemical catalysis. Very recently, it has been used for tailoring the specificities of enzymes, including α-amylases (Davis, 2003) due to the conserved nature of residues around the active sites of α-amylases, and the close similarities between their three dimensional structures (Machius *et al*, 1995; MacGregor *et al*, 2001). In a number of cases, dramatic stabilization has been achieved (Miland *et al*, 1996). The results obtained provide information of general utility for various α-amylases including those with unusual mechanism of action (Ammar *et al*, 2002). Enzymes with new catalytic activities can be created, and the specificities of existing enzymes can be changed to accommodate unnatural substrates. This relatively simple procedure would complement other techniques such as site-directed mutagenesis used for structural modification of a protein.

One of the five flexible regions in BLA (*Bacillus licheniformis* amylase) (residues 440 to 448), consists of the sequence -Arg-Gln-Asn-. This sequence in BAA (*Bacillus amyloliquefaciens* amylase) contains a lysine instead of glutamine. The asparagine in the position of lysine in the protein contributes to its deamidation (Wright, 1991). The modification of this lysine residue could lead to the lower extent of deamidation observed at 80°C in case of BLA. A portion of the active site of BLA includes these three amino acids and lysine 234 (Habibi *et al*, 2004). Further, analysis of inactivation kinetics showed that modification of a single carboxyl group led to complete loss of the enzymic activity. The most likely amino acid with pH value 5 is an aspartic/glutamic carboxyl group. Confirmation of carboxyl-group involvement was sought by chemical modification with l-ethyl-3-(3-dimethyl amino propyl) carbodiimide, a reagent specific for carboxyl groups (Hoare and Khosland, 1967). The study by Kochhar and Dua (1984) indicated that a carboxyl group is indispensable for α-amylase catalysis, and modification of a single carboxyl group lead to complete loss

of enzymic activity. It was proposed by Ara *et al* (1995) that there could be possible involvement of histidine and serine residues which participate in the catalytic site of the alkaline amylase from alkalophilic *Bacillus* sp KSM-1378. A crucial involvement of tryptophan resiude(s) along with histidine was also suggested in the same species. In case of *B. flavothermus* α-amylase, selective modification of the histidine(s) was observed and there was no involvement of tryptophan, tyrosine, lysine or sulfhydryl residues (Bolton *et al*, 1997).This involvement of histidine was in accordance with other α-amylases reported for thermopiles *B.stearothermophilus* (Vihinen *et al*, 1990) and *B.caldovelox* (Bealin-Kelly *et al*, 1992) and mesophilic *B.amyloliquefaciens* and *B.subtilis* (Takase *et al*, 1992).

(iii) Fluorescence Spectroscopy: A tool for protein folding/unfolding study

Fluorescence spectroscopy is widely used to study peptides and proteins. The aromatic amino acids; viz tryptophan, tyrosine, and phenylalanine act as intrinsic fluorescent probes for studying protein conformation, dynamics, and intermolecular interactions. Of the three, tryptophan is the most popular probe. Tryptophan occurs as one or a few residues in most proteins and biologically active peptides. It is of great importance to study the folding, stability and aggregation of proteins. Biophysical studies can also help bridge biology and physics. The chemical reactions in the body are performed by proteins. Biophysical studies help in understanding the concept of functioning of proteins. The performance of biological functions in health and diseases can be predicted on these protein structures. The response to drug differs due to variations in structure and/or proteins. This property can be used in drug designing, diagnosis and disease control. Tailor made medicines can be made available for individual by taking an account of the susceptibility of an individual to the side effects. Biophysical studies help in proper understanding of protein folding and provides tools for treating diseases

Protein Folding: Protein folding is the process in which a linear polypeptide chain transforms into three-dimensional functional structure. A defined mechanism that governs folding remains a significant point in biophysics and molecular biology. The linear information of genetic message is translated into a three dimensional and functional structure of protein and this makes protein folding a topic of basic interest (Oas and Kim, 1988). A specific three-dimensional structure makes the protein

biologically active, which is common amongst all proteins. The genetic information is responsible for the primary structure of the protein and the linear sequence of amino acids in the polypeptide backbone. There is a spontaneous refolding exhibited by most of the protein in vitro after being completely unfolded. The three-dimensional structure must be determined by the primary structure. The reason behind this happening is yet unknown and this is 'the protein folding problem' (Creighton,1990).

Protein folding intermediates: A number of equilibrium and kinetic studies have lead to structural characterization of folding / unfolding intermediates. These intermediates are a prerequisite and in a way contribute to solving the folding problem. Partially folded states are characterized at equilibrium under mildly denaturing conditions, such as by altering pH, addition of salts and alcohols, chemical denaturants such as urea and guanidine hydrochloride or by changing temperature and pressure. The protein exhibits molten-globule like intermediate with rearranged secondary and tertiary structures and exposed hydrophobic amino acids on the surface (Kuwajima,1989).

Significance of protein folding : Proteins are the most important molecules found in living organisms. They are required and are used to support the skeleton, control senses, move muscles, digest food, defence against infections and process emotions. Christian Anfinsen in the early 1960's, showed that the proteins actually coil around themselves (Thomasson, 1996). If proteins become unfolded, they fold back into proper shape of their own accord without any requirement of a shaper or folder. There is a possibility wherein the protein will fold incorrectly. Proteins, suitably named as chaperones, keep their target proteins from getting off the right folding path. This explains the theory to protein folding diseases (King, 1993; Thomasson, 1996).

Variations in environmental conditions can significantly influence the folding and stability of a protein molecule. The structural aspects studied for α-amylase are, therefore, of significant interest to understand its structure–function relationship. The circular dichroism spectroscopic data revealed the native α-amylase *gt* to contain 25% α-helix, 21% β-sheet, and 54% random coils. Denaturation of α-amylase *gt* in presesnce of urea, at high concentration (8 M) enhances the exposure of the buried trp residues of the native α-amylase *gt* to the aqueous environment and thus showed low fluorophore. Fluorescence-quenching experiments using KI, CsCl, N-bromosuccinimide, and acrylamide exhibited interesting features of the tryptophan microenvironment. Analysis

of K_{sv} and f_a values of KI, CsCl, and acrylamide suggested the overall trp microenvironment in α-amylase to be slightly electropositive. Both collisional as well as static quenching processes were observed on fluorescence-quenching studies with acrylamide. The observations presented in this communication provide some mechanistic features related to the role of aromatic trp moieties and the positive nature of the microenvironment around the trp moieties in stabilizing the hyperthermostable, Ca^{2+} independendent, and high-maltose-forming α-amylase of *G. thermoleovorans*. (Rao and Satyanarayana, 2008).

A low-pH-induced molten globule (MG) state was described for α-amylases from mesophilic and thermophilic species of *Bacillus* (Shokri *et al*, 2006). Arginine residues have also been linked to the ability to facilitate the formation of an MG state (Xie *et al*, 2004). Most studies focusing on improving protein stability have investigated the effects on the native state, and little attention has been directed at the unfolded state or MG state. Generally it is assumed that the unfolded state possesses no meaningful structure but it was found that TAA_{MOD} (Taka alpha amylase) had formed a thermostable, MG state. (Siddiqui *et al*, 2010).

Far-UV circular dichroism analysis revealed that the secondary structures of *Bacillus* sp. TS-23 α-amylase were significantly altered in the presence of various metal ions and SDS, whereas acetone and ethanol had no unfavourable effect on folding of the enzyme. *Bacillus* sp. TS-23 α-amylase was inactive and unstable at extreme pH conditions. Thermal unfolding of the enzyme was found to be highly irreversible. The native enzyme started to unfold beyond 0.2 M guanidine hydrochloride (GdnHCl) and reached an unfolded intermediate. *Bacillus* sp. TS-23 α-amylase was active at the concentrations of urea below 6 M, but it exhibited an irreversible unfolding by on incubating in 8 M denaturant. The folding and unfolding reveals the structure function relation which poses numerous advantages of using enzymes in organic solvents, such as the increased solubility of nonpolar substrates and the elimination of microbial contamination in the reaction mixture. The organic-solvent-tolerant characteristic studies help in predicting their suitability for biotechnological processes where the organic solvents are present. (Meng-Chun Chi *et al*, 2010).

(iv) Circular Dichroism

Circular dichroism (CD) refers to the differential absorption of left and right circularly polarized light.(Atkins and de Paula, 2005; Edward and Solomon, 2006). This phenomenon was discovered by Jean-Baptiste Biot, Augustin Fresnel, and Aimé Cotton

in the first half of the 19th century (Fasman 1996). CD spectroscopy has a wide range of applications in many different fields. Most notably, UV CD is used to investigate the secondary structure of proteins.(Nakanishi *et al*, 1994) UV/Vis CD is used to investigate charge-transfer transitions (Neidig *et al*, 2007). Near-infrared CD is used to investigate geometric and electronic structure by probing metal d→d transitions (Edward and Solomon, 2006) Vibrational circular dichroism, which uses light from the infrared energy region, is used for structural studies of small organic molecules, and most recently proteins and DNA (Nakanishi *et al*, 1994). Effect of tin oxide nanoparticle binding on the structure and activity of *α*-amylase from *Bacillus amyloliquefaciens* has been studied (Khan *et al*, 2011). Proteins adsorbed on nanoparticles (NPs) are being used in biotechnology, biosensors and drug delivery.

The CD spectra exhibited characteristic peaks (intensive positive peak at around 190 nm and two negative double humped peaks at 208 and 222 nm) of a high *α*-helical content in native enzyme (Khosro *et al*, 2001). Far UV CD spectra of *Bacillus amyloliquefaciens* α-amylase suggest a relatively more compact structure shown by a more negative spectrum in far-UV CD and a relatively more rigid structure as indicated by higher values in the tryptophan region (290–305 nm) in the near-UV CD spectrum (Kelly and Price, 1997).

VI. Application of amylases

α-amylases along with cellulases represent the second largest group of currently used industrial enzymes. α-amylases constitute a class of of industrial enzymes having approximately 25% of the enzyme market (Rao *et al*, 1998). Enzymes from fungal and bacterial sources have contributed the most in industrial applications (Pandey, 2000). The probability of using actinomycetes specially, *Streptomyces* for enzyme production has been explored by Chandrasekaran (1987) and James (1991). Some important applications of amylases are described below.

(i) As detergent additives

Enzymes that have found wide application in the detergent industry include proteases, lipases, cellulases and amylases. Amylases not only enable removal of starchy stains, but also prevent the swollen starch from adhering to the surface of dishes and clothes (D'Souza *et al*, 2010). Such adhered starch acts as a glue for particulate soiling. 90% of all liquid detergents contain amylases (Gupta *et al*, 2003; Mitidieri, *et al*, 2006; Hmidet *et al*, 2009; D'Souza *et al*, 2010). Amylases that are (i) active at lower

temperatures (ii) active in alkaline pH (iii) stable in the presence of chelating agent ie Ca^{2+} independent amylase (iv) have oxidative stability and (v) are stable in the presence of proteases, are ideal as detergent additives (Kirk *et al*, 2002).

For several years the demand for α-amylases in laundry and automatic dishwashing detergents has also been growing (Upadek, 1997). However, amylases with pH optima between 5 and 7.5 (Yamamoto, 1988) are not useful in detergents, since the working pH range for detergent compatibility lies between 8 and 11 (Ito, 1997). Enzymes such as high alkaline pullulanase and alkali resistant neopullulanase and an alkaline isoamylase have been effectively used as additives in dishwashing and laundry detergents under alkaline conditions, especially when combined with α-amylase (Igarashi, 1992; Ara, 1992, 1993, 1995). Liquefying amylases from *Bacillus amyloliquefaciens* (BAA), *Bacillus stearothermophilus* (BSA) and *Bacillus licheniformis* have pH optima between 5 and 7.5 and hence they are not much preferred in detergents for laundry (Manning, 1961; Takasaki, 1985). The α–amylases from *Bacillus sp.* strain TSCVKK (Kanthi Kiran and Chandra, 2008), *B.licheniformis* NH1 (Hmidet *et al*, 2009), *B.amyloliquefaciens* MTCC (610) (Dahiya *et al*, 2010) and *A.niger* L119 (Mitidieri *et al*, 2006) have been shown to have detergent compatible properties and hence would have potential application as detergent additives.

Novozymes and Genencore International have engineered amylases for better performanve, oxidative stability and storage stability (Bisgaard-Frantzen *et al*, 1995; Tierny *et al*, 1995). Eg. Purafect OxAM and Duramyl.

ii) As a desizing agent in the textile industry

Amylases find application in the desizing process in the textile industries. Starch is first applied to the yarn to strengthen it before the fabric is woven. After the fabric is produced, α–amylases are appled to the fabric to selectively remove the starch (Hendriksen, 1999; Gupta *et al*, 2003).

iii) As a sizing agent in the paper industry

Low viscosity, high molecular weight starch is used in the paper industry as a sizing agent (Bruinenberg *et al*, 1996; Van der Maarel *et al*, 2002; Gupta *et al*, 2003). As natural starch is highly viscous, α–amylases are used to partially degrade the starch for the coating treatment. This treatment makes the paper smooth and strong and also improves its quality and the coating treatment is carried out in a batch or continuous

process in the temperature range of 45°C – 60°C (Bruinenberg *et al*, 1996; Tolan *et al*, 1996). Examples of microbial α–amylase preparations used in the paper industry are Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes,Denmark) and amylase G9995® (Enzyme Biosystems, USA) (Gupta *et al*, 2003;Saxena *et al*, 2004).

iv) In the production of glucose and fructose syrup from starch

In the industrial production of glucose and fructose syrup from starch, the starch that is extracted from the plant source is first mixed with water and dissolved by using a jet cooker. The starch slurry is then passed through a hydrolytic reactor where the starch is degraded and converted into glucose and fructose by the action of three or four enzymes. α-amylases that are highly thermostable in the absence of metal ions are used in the first step to liquefy the starch and convert it to short chain dextrins. In the next step, enzymes such as glucoamylases, pullulanases and glucose isomerases are used for getting high fructose syrup (Van der Maarel *et al*, 2002).

v) In the baking industry

Starch degrading and starch modifying enzymes are widely used in the baking industry (Van der Maarel *et al*, 2002). The dough used for baking contains flour, water, salt, additives and yeast. The yeast ferments the sugars available in the dough to alcohol and carbon-dioxide which causes the dough to rise. α-amylases when added to the dough, degrade the starch into smaller dextrins which are fermented by the yeast. When the bread is stored, several changes such as loss of crispness of the crust, loss of flavor, increase in crumb firmness and loss of moisture of the crumbs occur which is called staling. Retrogradation of starch in the bread mainly contributes to the staling process (Kulp and Ponte, 1981; Champenois *et al*, 1999).To delay the staling process, several additives such as small sugars, emulsifiers, enzymes, etc, are added to the dough. These enzymes include branching enzymes, debranching enzymes, maltogenic amylases, β–amylases and amyloglucosidases (Van der Maarel *et al*, 2002). These enzymes are also used in clarifying the haze in fruit juices and beer and in the pre-treatment of animal feed to improve digestibility (Van der Maarel *et al*, 2002).

vi) In Biofuels

It is required to treat the starch-based grain with an alpha-amylase enzyme to convert the available starch to fermentable sugars in the production of ethanol. The article by Urbanchuk *et al* (2009) gives an account of the potential economic and environmental benefits of Corn Amylase on the production of ethanol from corn and sorghum. The total energy balance for corn to ethanol has been reported to be in the range from 0.54 to 2.10 (Wang, 2005). Different results from analyzing the carbon footprint of corn-based ethanol have been found by various researchers. Ethanol from corn has been found to minimize greenhouse gas (GHG) emissions by as much as 60%, when compared with gasoline. Whereas others have reported that it may increase carbon emissions over gasoline by as much as 20% (Wang, 2005). It has recently been investigated that α–amylase from *B. subtilis* is a potential source for producing fermentable sugars from the left-overs of sweet potatoes. These fermentable sugars will be bioconverted into ethanol as a source for biofuels. (Inman *et al*, 2010).

vi) Applications of malto-oligosaccharides forming α–amylases in the food and pharmaceutical industries

The concept of functional foods ie foods or food ingredients that have a beneficial effect on human health, is one of the leading trends in today's food industry. In this context, probiotics ie living microbial food supplements and prebiotics ie non-digestible food ingredients have received increased attention in recent times (Rastall and Maitin, 2002; Siro *et al*, 2008). Prebiotics are mostly oligosaccharides containing between 3 and 10 sugar moieties or simple sugars. The oligosaccharides commonly used in the food and beverge industry include galacto-oligosaccharides, lactulose, lactosucrose, fructo-oligosaccharides, palatinose or isomaltose oligosaccharides, glycosyl sucrose, malto-oligosaccharides, cyclodextrins, gentiooligosaccharides and xylo-oligosaccharides (Prapulla *et al*, 2000). Prebiotics that are produced industrially by enzymatic approach are oligofructose, fructooligosaccharides, lactosucrose, galactooligosaccharides, maltooligosaccharides, isomaltooligosaccharides and xylooligosaccharides (Casci and Rastall, 2006). Maltooligosaccharides are not produced by all α-amylases but are produced by only a few α-amylases which specifically act on starch to produce malto-oligosaccharides with DP 3-7.

Interest in the use of malto-oligosaccharides as biopreservatives and functional foods has grown in recent years (Crittenden and Playne, 1996; Barreteau *et al*, 2006).

Traditionally, malto-oligosaccharides which are the products of starch liquefaction have a degree of polymerization (DP) between 2 and 10 (G2-G10). Since these oligosaccharides are natural components of foods such as fruit, vegetables, milk and honey, they are increasingly being favoured in lieu of chemical additives which are becoming less welcome by the consumers (Rivero-Urgell and Santamaria-Orleans, 2001; Barreteau *et al*, 2006; Mussatto and Mancilha, 2007) Amongst the malto-oligosaccharides, G3 ie maltotriose, G4 ie maltotetraose, G5 ie maltopentaose and G6 ie maltohexaose have attracted attention because they not only provide useful modifications to flavour and physiocochemical characteristics of the food, but also have properties that are beneficial to human health (Crittenden and Playne, 1996; Rivero-Urgell and Santamaria-Orleans, 2001; Barresi *et al*, 2003; Barreteau *et al*, 2006). Some of the properties of these malto-oligosaccharides such as their high solubility, high viscosity, low sweetening power, non-hygroscopic nature, etc has encouraged their use in the food industry as binders, fat sustitutes, texturizing agents and thickeners (Barresi *et al*, 2003). Major uses of maltooligosaccharides are in beverages, infant milk powders, confectionery, bakery products, yoghurts and dairy desserts.

Properties of maltooligosaccharides with DP 3-7

(i) Maltooligosaccharides syrup especially G3/G4 strup syrup has several advantages compared to high fructose syrup or glucose. This low sweetness of G3 and G4 is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors. G3 and G4 are only 0.3 times sweet as compared to sucrose or high fructose corn syrup This property is an advantage when the aftertastes produced by some of the intense sweeteners are required to be masked (Mussatto and Mancilha, 2007). Maltotetraose and its stable reduced form maltotetraitol are used in food products as sweeteners with moderate sweetness, to control viscosity, to retain flavour, to impart gloss, to prevent crystallization and stickiness. Compared with mono- and di-saccharides, the higher molecular weight of oligosaccharides provides increased viscosity to the food products. (Crittenden and Playne 1996; Barreteau, 2006). Hence these are used in foods and also to sweeten tobacco, cosmetics, toiletries, pharmaceuticals, lipstick, lipcream, medicines and as oral refreshing agents (Takasaki, 1985; Palacios, 2004)

(ii) Like other sugars, they provide 4 cal/g, but cause less osmotic pressure on ingestion. Coupled with their refreshing sweetness, easy digestability and low

osmotic effect, they are ideally suited for sports and health beverages (http://hayashibara-intl.com/food/food.htm). Takasaki (1986) described a method of using G-4 amylase to produce high maltotetraose and maltose content starch hydrolysates. Novozymes A/S (2000) describes the enzymatic preparation of maltose-maltotriose starch hydrolysates (Leach *et al*, 1978).

(iii) They are more resistant to heat than glucose and fructose and hence the amount of browning due to Maillard reactions in heat-processed foods can be controlled (Mussatto and Mancilha 2007).

(iv) They have a low water activity due to which microbial contamination can be prevented (Okada and Nakakuki, 1992).

(v) They are highly soluble and produce clear viscous solutions that are palatable (Kobayashi, 1990).

(vi) They are non-cariogenic and hence can be used in foods and beverages. (Barreteau, 2006; Mussatto and Mancilha, 2007)

(vii) They alter the freezing temperature of frozen foods and hence are useful in increasing the shelf life of frozen foods.

(viii) They also have a higher moisture-retaining capacity and thus can prevent excessive drying.

(ix) They prevent retrogradation of starch and act as antistaling agents (Dziezak, 1991; Martin and Hoseney, 1991; Okada and Nakakuki, 1992; Park, 1992; Crittenden and Playne, 1996; Min, 1998; Duran, 2001; Gray and BeMiller, 2003; Smits, 2003; Nakakuki, 2003). Hence, they have been shown to increase the shelf life of bread and baked products. Patent WO/2000/059307 (Nielsen and Schaefer, 2001) describes the preparation of dough and baked products using an amylase which hydrolyses starch to form maltotriose which helps to retard staling of bread.

The maltooligosaccharides with DP 3-7 have been shown to have the following health benefits.

(i) Maltotetraose enrichned corn syrup was shown to inhibit the growth of intestinal putrefactive bacteria such as *C. perfringens* and Enterobacteriaceae and hence was effective in improving colonic conditions (Okada and Nakakuki, 1992; Kubota *et al*, 1999).

(ii) The maltooligosaccharides selectively induce the growth and activity of one species or a limited number of activities of one species or a limited number of species of

bacteria in the colon. These functional oligosaccharides are usually not absorbed in the small intestine. They are fermented in the colon and hence provide less energy than carbohydrates.

(iii) Maltooligosaccharides serve as the starting compounds for the production of isomaltooligosaccharides, which are specifically metabolized by the beneficial bacterial microflora (*Bifidobacterium*, *Bacteroides*, *Lactobacillus* genera). Isomaltooligosaccharides prevent dental cavities and act as immunostimulant substances (Kaneko,1994; Duan,1995; Barresi,2003)

(iv) The saccharide compositions consisting maltohexaose and/or maltoheptaose or containing maltohexaitol and/or maltoheptaitol can be also used as feeds and pet foods for domestic animals, poultry and fishes. It improves their taste preferences. These sweetners can be arbitrarily used as a sweetner, taste improving agent or quality improving agent in other products. The range of products with these sweetners are in the form of a solid paste or liquid such as tobacco, cigarette, dentrifice, lipstic, rouge, lipcream, internal medicine, tablet, troche, cod liver oil in the form of drop, cachow, oral refrigerant, gargle, cosmetics or pharmaceutical (Nakano *et al*, 2001).

(v) The saccharide G6/G7 or those containing maltohexaitol and/or maltoheptaitol can be used as quality improving agent or a stabilizer for biologically active substances. (Nakano *et al*, 2001).

Nihon Shokuhin Kako (Japan Maize Products, Tokyo) and Hayashibara Shoji Inc (Japan) are the largest producers of malto-oligosaccharides in Japan (Crittenden and Playne 1996). The market for these oligosaccharides in Japan alone was projected to be more than 20 billion yen/year (Nakakuki, 2003). Though Japan is the major producer of oligo-saccharides, the use of different oligosaccharides by food manufacturers outside Japan is also increasing (Crittenden and Playne, 1996).

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Chapter 2

Characterization of the *Streptomyces* strain NCL 716

Summary

An alkalophilic actinomycete designated as strain NCL 716 was isolated during a regular screening programme, from the soil surrounding Lonar lake, a salt water meteorite lake, in Buldhana district in the State of Maharashtra in India. This strain was found to be a facultative alkalophile as it grew optimally at 28°C and in the pH range of 8.0 to 12.0. It was also found to secrete an alkaline malto-oligosaccharide producing amylase.

This chapter describes the identification and characterisation of this actinomycetes strain, using a polyphasic approach which included rDNA analysis, classical and chemotaxonomical approaches. This strain was identified as a Streptomyces strain and was given the name Streptomyces lonarensis strain NCL 716 as it was isolated from the soil around Lonar lake. The rDNA sequence was submitted to the GeneBank and was assigned the GenBank accession number FJ919811.

Introduction

The genus *Streptomyces* is one of the best studied and characterized genera of the order Actinomycetales and family Streptomycetaceae because of the production of a large collection of biologically useful compounds by the members of this genus. These include antibiotics, anti-parasitic agents, herbicides, immuno-suppressants and several enzymes useful in the food and other industries. (Embley and Stackebrandt, 1994; Bentley *et al*, 2002; Cerdeno-Tarrager *et al*, 2003; Hopwood, 2007). More than half of the known antibiotics are derived from actinomycetes of which 75% are made by *Streptomyces* (Berdy, 2005). Many members of this genus also secrete enzymes important in biodegradation and bioremediation (Phelan *et al* 1979; Grund *et al* 1990; Ishiyama *et al*, 2004).

The genus Streptomyces earlier known as Streptomytaceae was proposed by Waksman and Henrici (1943) (Waksman et al, 1943) to describe bacteria that are mainly aerobic, Gram positive and non-acid fast actinomycetes. The identification, classification and characterisation of Streptomyces has relied mainly upon classical, chemotaxonomical and molecular approaches:

(1) Classical approach: In this approach, morphological, physiological and biochemical characters are used to identify and characterize a strain nm (Shirling and Gottleib, 1966; Buchanan and Gibbons 1974; Nonomura, 1974; Anderson and Wellington, 2001; Burkholder et al, 2006).

Morphological characters include:

(i) Morphology of the colonies: The shape, size, texture and colour of the colonies grown under uncrowded concentrations on 'standard' media is used as an important character for identification and classification of an isolate.

(ii) Pigments: The pigments observed in aerial and substrate mycelium in the vegetative growth, in the spore masses and in the medium around the colonies has been used as a criterion for identification by Krassilnikov, 1941; Waksman and Henrici, 1948; Hesseltine,1954 and Lindenbein,1952).

(iii) Morphology of the aerial mycelium i.e whether the mycelium consists of short or long hyphae, type of branching of the mycelium ie whether simple or extensively branched and if branched the type of branching ie monopodial, sympodial, verticillate or irregular.

(iv)Morphology of the sporophore. Pridham et al (1957, 1958) proposed subdivision of the genus Streptomyces into seven morphological; sections based on the structure of the

sporophore ie Rectus-Flexibilis (RF), Retinaculum-Apertum (RA), Spira (S), Monoverticillus (MV), Monoverticillus-Spira (MV-S), Biverticillus (BIV) and Biverticillus-Spira (BIV-S).

(v) Morphology of the spore: This description includes

- the shape and size of the spores which ranges from globose to elliptical to elongate to cylindrical; and
- ornamentation on the spore surface.

Tresner *et al* (1961) classified the spores into four morphological types i.e smooth, warty, spiny and hairy.

Physiological and biochemical characters (Waksman, 1961; Burkholder *et al* 2006)

Some of the important biochemical and physiological properties that have been used in the identification of a strain and in the classification of *Streptomyces*, include

(i) Utilization of carbon sources: Growth and development of a *Streptomyces* strain on a basal medium having different carbon compounds has been used in the characterisation and identification of *Streptomyces* strains (Pridham and Gottleib, 1948). The carbon sources used include cellobiose, galactose, maltose, mannitol, levulose, arabinose, rhamnose, sodium succinate, xylose, lactose, inositol, salicin, sucrose, sodium citrate, raffinose, inulin, sorbitol, dulcitol and cellulose.

(ii) Utilization of nitrogenous sources: Utilization of certain nitrogenous compounds such as glutamic acid, aspartic acid, ammonium chloride, glycine, sodium nitrate, etc by the strain has also been used as a basis of characterization. (Shinobu, 1958; Burkholder *et al*, 2006).

(iii) Production of antibiotics and inhibitory properties: The spectrum and range of activity of the antibiotics produced by a particular strain, together with their physical and chemical properties, provides a basis for the identification and characterization of a strain (Burkholder *et al,* 2006). The different pattern of susceptibility and resistance to a particular antibiotic is also used to identify and characterize a strain (Krassilnikov, 1950, 1958, 1960a and 1960b; Gausse 1955; Burkholder *et al,* 2006).

(iv) Sensitivity to actinophages: Actinophages are known to attack certain strains of actinomycetes resulting in lysis and formation of a typical plaque (Korn *et al*, 1978; Burkholder *et al*, 2006). Such sensitivity to actinophages is used to characterize a strain.

Actinophages can be used for host-identification at the genus and the species level (Wellington and Williams, 1981).

(v) Serum diagnosis: Immunological techniques based on agglutinin and precipitin techniques have been developed by Ludwig and Hutchinson (1949) as a basis for identification of relationships in actinomycetes. The use of antisera in *Streptomyces* taxonomy was investigated by Ridell *et al* (1986) and Kirby and Rybicky (1986). However, this approach did not have the reproducibility and specificity of monoclonal antibodies. Wipat *et al* (1994) used monoclonal antibodies specific for *S. lividans*. However, there are certain limitations in using monoclonal antibodies to classify soil Streptomycetes (Nelsen *et al*, 1991; Smith *et al*, 1991).

(vi) Hydrolytic reactions: The ability of a *Streptomyces* strain to secrete enzymes which act on certain substrates is used to characterize the strain. Examples include degradation of starch and gelatin, inversion of sucrose, lipolysis, etc (Burkholder *et al*, 2006).

(vii) Formation of hydrogen sulfide in peptone-iron-agar medium has been used to characterize *Streptomyces* strains (Tresner and Danga 1958)

(viii) Growth characteristics of the culture at different pH and temperatures and also in the presence of different amounts of NaCl is yet another characteristic which is used to describe a strain (Shirling and Gottleib, 1966).

(2) Chemotaxonomical approach: This approach includes estimation of whole cell sugars, analysis of cell wall amino-acids, analysis of polar lipids, isoprenoid quinones and analysis of cellular fatty acids and phospholipids in membranes (Gochnauer et al,1975; Collins et al,1977; Lechevalier et al,1977; Goodfellow and Cross,1984; Kroppenstedt, 1985; Embley et al,1986, 1987).

The presence of DAP (diaminopimelic acid) isomers is one of the characteristics of the cell wall of gram positive bacteria and actinomycetes. The cell wall of actinomycetes is categorized into nine types i.e I to IX depending upon the presence of the DAP isomer (either LL DAP or meso/DL DAP) and the presence and absence of some diagnostic amino-acids (Lechevalier and Lechevalier, 1970). Members of Streptomyces have cell wall type I (LL DAP and no characteristic sugar (Lechevalier and Lechevalier, 1970).

The application of chemical methods has influenced the development of actinomycete classification at the genus level and even at higher taxonomic levels (Lechevalier and Lechevalier, 1970; Minnikin et al, 1980; Goodfellow *et al*, 1984; Kroppesnstedt, 1985; Stackerbrandt, 1986; Saddler et al, 1987; Vandamme et al, 1996; Kim et al, 1998; Atalan et al, 2000; Sahin et al. 2001). Lipid profiles typically contain hexa and octa-hydrogenated menaquinones with nine isoprene units as the predominant isoprenologues. The polar lipid profiles are composed of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides. Fatty acids are complex mixtures of saturated, *iso*- and *anteiso*-fatty acids. Mycolic acids are not present in Streptomyces.

(3) Numerical Classification: Numerical classification is based on classifying the strains based on the simultaneous evaluation of several morphological, physiological, biochemical, chemotaxonomical and other characteristics. Numerical taxonomy has been applied to Streptomycetes since the 1960s (Silvestri et al, 1962; Gladek et al, 1985; Christova et al, 1995).

Silvestri *et al* (1962) studies 200 strains on the basis of 100 characters. Williams *et al* (1983 a, b) used 139 characteristics to classify 394 Streptomycetes type cultures. His study formed the basis of classification of the species of the genus *Streptomyces* in Bergey's manual The basic data of the numerical taxonomy of Williams (1983 b) are used to create a probabilistic identification matrix which is used in the identification of unknown Streptomycetes as well as for the identification of the species group defined by the numerical taxonomy (Williams *et al*, 1983b; O'Donnell, 1988).

Numerical taxonomy, however, does not consider the phylogenetic linkages among the bacteria and hence it cannot clearly distinguish between closely related species. The phenotypic, chemical, enzymatic and serological characteristics account for only 5-19% of the genome. Molecular methods such as protein patterns, MLEE analysis, restriction analysis, plasmid patterns, rDNA analysis, DNA fingerprinting with different probes and primers would give a more precise estimate of the genetic relatedness among species (Christova *et al*, 1995).

4) DNA-DNA Hybridization: DNA-DNA hybridization technique is also valid in *Streptomyces* classification at the genus level (Anderson *et al*, 2001). In order to determine the species identity within *Streptomyces*, DNA-DNA hybridizations of the total chromosomal DNA have been used. The reassociations of ssDNAs have been used. The degree of relatedness is given in terms of % homology and the genomic definition of species is suggested to include strains with 70 % DNA-DNA relatedness and 5°C differences in the melting point temperature between the homologous and heterologous hybrids formed. The hybrid is formed wherein standard stepwise denaturisation conditions are used (Wayne *et al*, 1987).

(5) Molecular approach based on rDNA analysis: The sequence of the 16S rDNA gene has been extensively used not only to study the phylogeny of bacteria but also to dentify an unknown bacterium upto the genus or species level (Sacchi et al, 2002) The 16S RNA gene sequence has properties which have made it a universal phylogenetic marker. It has regions which are evolutionarily conserved and others which are variable. Since the rRNAs are essential, they are highly conserved throughout different species, lateral transfer is very rare and their molecular size ensures that they carry abundant evolutionary informative sites. The varying degree of sequence conservation allows reconstruction of phylogenies for a broad range of relationships upto the species level. Comparing the difference in the base sequence of the 16S rRNA gene is therefore an excellent phylogenetic tool to study the phylogenetic relationships amongst organisms (Woese, 1987; Olsen *et al*, 1993; Ludwig and Schleifer, 1994). Since each position in the sequence carries information on a very narrow range of evolutionary time, a large number of independently evolving positions leads to a better phylogenetic resolution (Olsen, 1993). The comparison of rRNA sequence has proved to be a powerful tool in Streptomycete taxonomy. The α and β variable regions have been useful for genus specific classification, while the γ variable region has been useful for species-specific classification within the *Streptomyces* genus. (Stackebrandt *et al*, 1991a, b, 1992; Anderson and Wellington, 2001**).**

General description of *Streptomyces* species: *Streptomyces* species produce vegetative hyphae 0.5 - 2.0 μm in diameter which forms an extensively branched

mycelium which rarely fragments. This matures to form chains of three to many non-motile spores. Members of a few species bear short chains of spores on the substrate mycelium. Cells are Gram-positive but not acid-alcohol fast. Growth is obligately aerobic and the optimum growth temperature is 25°C – 35°C. Initially the colonies produced are relatively smooth surfaced but later they develop aerial mycelium which may appear floccose, granular, powdery or velvety. Colonies are discrete, lichenoid, leathery or butyrous. The vegetative and aerial mycelia may be pigmented and diffusible pigments may also be produced. The various species grow over a wide pH range from pH 5 to 11.5. The cell wall is of type I i.e it has LL-diaminopimelic acid and no characteristic sugar. The various members differ in their ability to utilise the different carbon and nitrogenous sources.The DNA base composition ranges from 66 to 73 mol% of G+C (Pridham et al, 1974).

Materials and Methods

Materials

Chemicals:

Chemicals, media components etc. were purchased from various Companies as described below:

Hi-Media (Mumbai, India):

Agar, Beef extract, D-glucose, Malt extract, Peptone, Tryptone, Yeast extract, Yeast Nitrogen Base (YNB) etc.

Loba chemicals (India):

K_2HPO_4, MgSO4.7H_2O, $FeSO_4$.7H_2O, $ZnSO_4$.7H_2O, $NaNO_3$, $MnCl_2$, NaCl, $(NH_4)_2SO_4$

MERCK, Sisco Research Laboratories (SRL) (India):

Absolute ethanol, Acetic acid, Agarose, Glycerol, KCl etc.

Qualigens Fine Chemicals (India):

Glycerin, $MgCl_2$, Potassium sodium tartarate, Sodium carbonate, Sodium hydroxide.

Other chemicals used were aspargine, $(NH_4)_2SO_4$, sodium propionate, soluble starch, tyrosine, etc.

Plasticware:

Following plastic ware was procured from Tarsons, Axygen and Laxbro (India).

Centrifuge tubes, disposable Petri dishes, Eppendorf tubes, micro-pipette tips, micropipettes from Tarsons and Thermo Electron Corporation.

Glasswares

Following glassware were purchased from Borosil or Corning Ltd (India): Conical flasks, glass- beakers, measuring cylinders, spreaders, test tubes, thermometer.

Instruments

Following equipment were used to carry out day to day experiments:

Autoclave (Ultradent, India); magnetic stirrer and centrifuge (Remi Scientific Instruments, India); electrophoresis apparatus (Tarsons, India); gas burner (local make); Pierce Reacti-Therm heating block (Pierce Chemical Co., USA); ice machine, laminar air flow (Micro-Filt, India), microwave oven, pH meter, power pack, shaker-incubator, Spectrophotometer (Shimadzu), thermostat water bath (Tarsons), Sonicator (Misonix), -20^0C freezer (Cellfrost, India) and -70^0C freezer (Dairei).

Media composition

Czapek media:

Glucose - 13%

KH_2PO_4 - 1.0%

KCl - 0.3%

$MgSO_4.7H_2O$ - 0.3%

$FeSO_4.7H_2O$ - 0.03%

$ZnSO_4.7H_2O$ - 0.03%

$NaNO_3$ - 0.6%

Agar - 3.0%

Glycerol-aspargine agar (ISP 5)

L-Aspargine - 0.1%

K_2HPO_4 - 0.1%

Trace salt solution - 0.1%

Glycerol - 1.0%

Agar - 2.0%

Inorganic salt-starch agar (ISP 4)

Soluble starch - 1.0%

K_2HPO_4 - 0.1%

$MgSO_4$ - 0.1%

NaCl - 0.1%

$(NH_4)_2SO_4$ - 0.2%

$CaCO_3$ - 0.2%

$FeSO_4$ - 0.0001%

$MnCl_2$ - 0.0001%

$ZnSO_4$ - 0.0001%

Agar - 2.0%

MGYP (Malt Extract Glucose Yeast Extract Peptone)

Malt extract - 0.3%

Glucose - 1.0%

Yeast extract - 0.5%

Peptone - 0.5%

Agar - 2.0%

Nutrient agar:

Beef extract - 0.3%

Peptone - 0.5%

Agar - 1.5%

Oatmeal agar (ISP 3)

Oatmeal - 6%

Agar - 2.5%

PDA (Potato Dextrose Agar):

Potato - 20%

Dextrose - 2.0%

Agar - 2.0%

PSA (Potato Starch Agar):

Yeast extract - 0.5%

Peptone - 0.5%

Potato starch (soluble) - 3.0%

K_2HPO_4 - 2.0%

$MgSO_4$ - 1.0%

Tyrosine agar (ISP 7)

Peptone - 0.5%

Beef extract - 0.3%

Agar - 2.0%

L-Tyrosine - 0.5%

Yeast extract agar (ISP 2)

Yeast extract - 0.4%

Peptone - 1.0%

Dextrose - 0.4%

Agar - 2.0%

Trace salt solution

$FeSO_4$ - 0.1%

$MnCl_2$ - 0.1%

$ZnSO_4$ - 0.1%

Cultures used to test the antimicrobial activity of strain NCL 716

The following cultures required for the study of antimicrobial activity of strain NCL 716 were procured from NCIM (The National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India):

(i) Bacterial cultures: *Bacillus subitilis NCIM 2063, Escherichia coli NCIM 2065, Pseudomonas aeruginosa NCIM 2200, Pseudomonas fluorescens NCIM 1099, Staphylococcus aureus NCIM 2079.*

(ii) Fungal cultures *Candida albicans NCIM 3471, Cuvularia lunata NCIM 716, Fusarium proliferatum NCIM 1103.*

Methods

(I) Isolation of the organism

The soil sample used for isolation was collected from the vicinity of Lonar lake, a meteorite salt water lake situated in Buldhana district of Maharashtra State, India. The protocol and media for isolation of alkalophilic actinomycetes was as described by Mikami *et a.* (1982). The pH of the medium was adjusted to 10.5 by addition of sterile Na_2CO_3, prior to inoculation. Purification of the isolated cultures was carried out by the dilution technique repeatedly to overcome bacterial contamination. No antibiotics were used.

(II) Identification and phylogenetic analysis by 16S rDNA analysis

DNA was extracted from the culture using the protocol of Wang *et al*. (2005). The 16S rDNA was amplified using eubacterial specific primers 27f (5′AGAGTTTGATCMTGGCTCAG-3′) and 1488r (5′-CGGTTACCTTGTTACGACTTCACC-3′). The PCR conditions used included an initial denaturation at 94°C for two min., followed by 35 cycles of denaturation at 95°C for one min., annealing at 55°C for one min. and extension at 72°C for one min. followed by a final extension at 72°C for 10 min. The sequence of the 16S rRNA gene was subjected to a BLAST search at the NCBI site (http://www.ncbi.nlm.nih.gov). The 16S rDNA sequences of first 100 hits which showed maximum homology with the *Streptomyces* species NCL strain 716 were selected and these sequences were aligned using ClustalW2. Rooted phylogenetic trees were constructed using Neighbor-Joining method, using the program TreeView X 0.5.1. (http://treeview-x.en.softonic.com/)

(III) Study of the growth characteristics at different pH, temperature and NaCl concentration.

(i) Effect of temperature on growth

The *Streptomyces* strain was allowed to grow in PSA medium at pH 9.0, incubated at different temperatures i.e. 28^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 50^{o}C for one week, after which the growth characteristics were noted.

(ii) Effect of pH

The growth of the *Streptomyces* strain was observed by varying the pH of basal medium (PSA) from pH 4.0 to pH 11.0) at 28^{o}C for one week.

(iii) Effect of NaCl on growth

Varying concentration of NaCl (1% - 10%) was added to the basal medium at pH 9.0 and the plates were incubated at 28^{o}C for one week.

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(IV) Morphological, physiological and biochemical characterization of the actinomycete strain NCL 716

To study the cultural, morphological and physiological characteristics, the strain was grown in different media. Media used for the study include aspargine agar, czapek media, glycerol-aspargine agar, inorganic salt-starch agar, nutrient agar, oatmeal agar, tyrosine agar, yeast extract agar and PDA. Observations were made over a period of three weeks and noted.

Morphological observations included noting the colour

(i) of the aerial and substrate mycelium

(ii) of the pigment formed in the plate.

Morphology of the sporophore and spores was observed by scanning electron microscopy as follows: The *Streptomyces* spores were streaked on the **P**otato **S**tarch **A**gar (**PSA**) plate and the plates were incubated for 10 and 21 days respectively. The spores were mounted by spreading them evenly on the carbon tape that was applied on the SEM stub.

Checking for Sugar utilization (utilization of carbohydrate source)

Spores were inoculated in 5 ml of 0.7% YNB (Yeast Nitrogen Base) without amino acids and 1% of sugar and incubated at 28^{o}C in a shaking incubator. The sugar solutions were autoclaved at 10 lb pounds for 10 min. and then YNB was added to make a final concentration of 0.7%. After every 24 h, tubes were checked for growth and the observations were noted.

Antibiotic resistance

The sensitivity of the strain NCL 716 to ampicillin, chloramphenicol, gentamycin sulphate, kanamycin sulphate, nalidixic acid, novobiocin sulphate, nystatin, refampicin, tetracycline hydrochloride and vancomycin hydrochloride was determined as follows: A spore suspension in 0.01% Tween 80 of a 7-day-old culture on PSA slant was made. 50 µl of spore (2×10^7) suspension was spread on LB plates. After spreading, immediately three wells were bored in each plate. In each well different concentrations of the particular antibiotic was added. Concentrations taken were 10 µg, 30 µg, 60 µg, 90 µg, 120 µg, 150 µg, 180 µg, 210 µg and 250 µg. After addition of the antibiotics, plates were incubated at 28^{o}C. The results were noted in the form of zone of clearance after 48 h.

Antimicrobial activity

Antimicrobial activity of the cell free extract of the NCL 716 strain was studied on the following microorganisms:

(i) Bacterial cultures: *Bacillus subitilis NCIM 2063, Escherichia coli NCIM 2065, Pseudomonas aeruginosa NCIM 2200, Pseudomonas fluorescens NCIM 1099, Staphylococcus aureus NCIM 2079*

(ii) Fungal cultures *Candida albicans NCIM 3471, Cuvularia lunata NCIM 716, Fusarium proliferatum NCIM 1103.*

The following procedure was used to study the antimicrobial activity of the strain: A spore suspension in 0.01% Tween 80 was made from a 7-day-old PSA slant. 1 ml of this spore suspension was inoculated into 50 ml inoculum media in a flask. The flask was kept shaking at 180 rpm for 48 h in an incubator set at 28°C. After 48 h, 5 ml of inoculum was added into 50 ml fermentation media and the flask was kept in an orbitary shaker at 28^{o}C at 160 rpm. After 108 h, the culture was harvested by centrifugation at 10,000 rpm for 10 min. and the broth was used as the cell free extract. The cell free extract was dialysed for 12 h with three changes of 10 mM Tris-HCl buffer (pH 7.6) and then lyophilized to concentrate the extract. The bacterial cultures were grown overnight in freshly prepared Nutrient Broth and the fungal cultures were grown in MGYP broth. These grown cultures were spread on the LB plates following Mcfarland 0.5 protocol. (McFarland, 1907). Wells were bored and the dialyzed, lyophilized cell free extract was added to the wells in increasing volumes. After addition

of the cell free extract in the wells the extract was allowed to diffuse and then the plates were kept for incubation at 28°C. The fungal culture *Cuvularia lunata* was incubated at 30°C. The antimicrobial activity was determined in terms of zone of inhibition after 48-72 h

Determination of amylolytic activity by plate assay

Determination of the amylolytic activity of the strain was carried out on agar medium in petri plates containing 1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.05% K_2HPO_4,01% $MgSO_4$ and agar 2.4% (PSA).The pH of the medium was adjusted to 10 by the addition of sterile Na_2CO_3 to a final concentration of 1%, prior to pouring the plates. The culture was inoculated at the center of the plates and incubated for 96 h at 28°C. The plates were then flooded with 0.1% iodine in 2% KI. Starch degrading activity was identified by a zone of clearance against a dark blue background.

(V) Chemotaxonomy studies

(i) Analysis of whole cell sugars and amino-acids

The procedure of Becker *et al*. (1965) was used to determine the whole-cell sugar and amino acid composition.

The cell wall hydrolysate was prepared as follows: The *Streptomyces* strain NCL 716 was grown in MGYP media in a shake flask at 28°C, and the cells were collected at maximal growth after 48 h. The cell mass was collected by centrifugation, and thoroughly washed with water. After treatment with ethanolic KOH (0.5%) at 37°C for 24 h, the cells were washed with ethyl alcohol until the pH was neutral, and were stored under ethyl alcohol at 4°C until used. 2-2.5 ml of the cell mass was sonicated (Misonix Sonicator) using the 4 mm probe, by pulses of 20 seconds for 45 min. The suspension was centrifuged at 1,200 g for 3 min to remove the glass beads and the unbroken cells. The supernatant was then centrifuged at 12,800 g, which caused a deposit of the crude broken cell walls. This deposit was washed twice with 0.05 M phosphate buffer (pH 8.0) followed by two washes with distilled water. The cell wall debris was resuspended in a freshly prepared and filtered solution of trypsin in phosphate buffer and digested at 37°C for 2 h on a rotary shaker. Trypsin was removed by washing twice with phosphate buffer and twice with distilled water. For further purification, a freshly prepared and filtered solution of pepsin in 0.02 N HCI was added to the walls and the mixture wasput on the shaker at 37°C overnight. The suspension was centrifuged, washed twice with

0.02 N HCI, twice with distilled water, and twice with 95% ethyl alcohol. The purified cell walls were dried in vacuum over H_2SO_4.

Hydrolysis and neutralization.

For amino acids and amino sugars, a 5 mg (dry weight) sample was hydrolyzed in 1 ml of 6 N HC1 in a sealed tube (13 X 100 mm) in a hot air oven at 100 ^{0}C for 18 h. The hydrolysate was filtered, evaporated to dryness three times on a boiling-water bath to remove the HC1, and dissolved in 0.3 ml of distilled water. The solution was filtered and then used for spotting.

For sugars: 10 mg (dry weight) sample was hydrolyzed in 1 ml of 2 N H_2SO_4 in a sealed Pyrex tube at 100^0C for 2 h. The hydrolysate was neutralized to pH 5.0 to 5.5. The precipitate of $BaSO_4$ was centrifuged at 3000 rpm and the supernatant fluid was poured off into a small beaker and evaporated to dryness in vacuum over H_2SO_4. The final product was redissolved in 0.4 ml of distilled water.

Chromatography: Amino acids and amino sugars were identified by spotting 10 µl of the hydrolysate on Whatman no.1 paper and descending paper chromatography. For detecting amino acids, the solvent system used for separation was methanol-pyridine-10 N hydrochloric acid- water (64:8:2:14, vol/vol) (Rhuland *et al*, 1955) After 20 h, the paper was removed, dried at room temperature. The paper was sprayed with 0.1% ninhydrin in acetone, followed by heating for 5 min or less at 100°C. Standard amino acids used were glutamic acid, lysine, leucine, glycine. A mixture of meso-α, ε-diaminopimelic acid, each at a concentration of 0.01 M, was spotted on the paper for comparison.

Detection of sugars was done using the solvent system comprising of n-Butanol-acetic acid-water 40: 10: 50 (Rhuland *et al*, 1955). The paper was dried and solution of phthalate anhydride in aniline was sprayed. The paper was further heated for 5 min at 100°C. Reference standards galactose, ribose, arabinose, mannose, glucose, xylose were run alongside.

(ii) Fatty acid analysis

Whole cell fatty acids were extracted and analyzed as methyl ester derivatives (FAMEs) as described by Kloepper *et al* (1992) and Miller and Berger (1985). FAMEs were analyzed with a Hewlett Packard Series II gas chromatograph Model 5890 equipped with a 25 m X 0.2 mm X 0.33 m phenyl methyl silicone capillary column. The

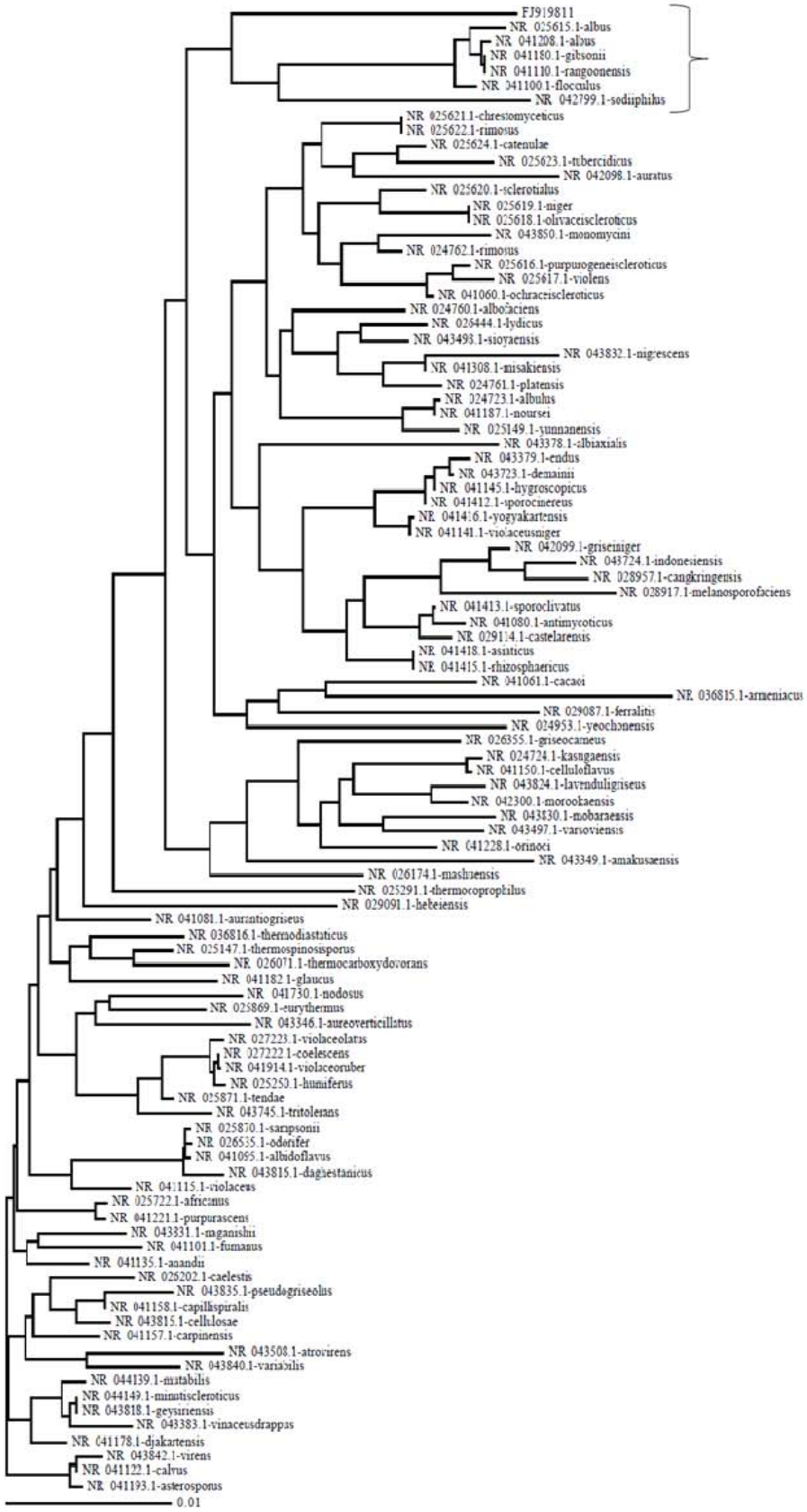
samples were processed using the Microbial Identification System MIDI (Newark, DE) which calibrates the gas chromatograph with a commercial FAME mixture (MIDI). The FAME profile of the sample to be analyzed is compared with the library entries using a pattern recognition algorithm that provides similarity indices of the organisms most similar to the sample that is being analyzed. The quantitative data obtained from the FAME profiles were used for numerical analysis wherein similarities were calculated with generalized similarity coefficient of Gower (1966) and the coefficient based on the Euclidean distance between pairs of strains (Boe and Gjerde, 1980).UPGMA dendrograms were generated using a program provided in the MIS Library Generation software (Sneath and Snokal, 1973).

Results and Discussion

(1) Isolation and identification of the organism

The actinomycete strain NCL 716 was isolated from the soil of the meteorite salt water Lonar lake, situated in Maharashtra, India, as described in `Materials and Methods'. The culture was identified to be a *Streptomyces* species based on the rDNA analysis of the complete sequence of 16s rDNA (Fig 2.1). The rDNA sequence was submitted to the GeneBank and was assigned the GeneBank accession number FJ919811. The strain was named as *Streptomyces lonarensis* strain NCL 716.

***Fig 2.1*: Neighbour-joining phylogenetic tree of the nearly complete 16S rDNA sequence of *Streptomyces lonarensis* strain NCL 716 (FJ 919811) and representative strains of the genus *Streptomyces*.**


FJ919811
NR 025615.1-albus
NR 041208.1-alvus
NR 041180.1-gibsonii
NR 041110.1-rangoonensis
NR 041100.1-flocculus
NR 042799.1-sodiphilus
NR 025621.1-chrestomyceticus
NR 025622.1-rimosus
NR 025624.1-catenulae
NR 025623.1-tubercidicus
NR 042098.1-auratus
NR 025620.1-sclerotialus
NR 025619.1-niger
NR 025618.1-olivaceisclerotícus
NR 043850.1-monomycini
NR 024762.1-rimosus
NR 025616.1-purpusogeneisclerotícus
NR 025617.1-violens
NR 041060.1-ochraceisclerotícus
NR 024760.1-albobiciens
NR 026444.1-lydicus
NR 043493.1-sioyaensis
NR 043832.1-nigrescens
NR 041308.1-misakiensis
NR 024761.1-platensis
NR 024723.1-albulus
NR 041187.1-noursei
NR 025149.1-yunnanensis
NR 043378.1-albiaxialis
NR 043379.1-endus
NR 043723.1-demainii
NR 041145.1-hygroscopicus
NR 041412.1-sporocinereus
NR 041416.1-yogyakartensis
NR 041141.1-violaceusniger
NR 042099.1-griseiniger
NR 043724.1-indonesiensis
NR 028957.1-cangkringensis
NR 028917.1-melanosporofaciens
NR 041413.1-sporoclivatus
NR 041080.1-antimycoticus
NR 029114.1-castelarensis
NR 041418.1-asiaticus
NR 041415.1-rhizosphaericus
NR 041061.1-cacaoi
NR 036815.1-armeniacus
NR 029087.1-ferralitis
NR 024953.1-yeochonensis
NR 026355.1-griseocameus
NR 024724.1-kasugaensis
NR 041150.1-cellulofiavus
NR 043824.1-lavenduligriseus
NR 042300.1-morookaensis
NR 043830.1-mobaraensis
NR 043497.1-varioviensis
NR 041228.1-orinoci
NR 043349.1-amakusaensis
NR 026174.1-mashuensis
NR 025291.1-thermocoprophilus
NR 029091.1-hebeiensis
NR 041081.1-aurantiogriseus
NR 036816.1-thermodiastaticus
NR 025147.1-thermospinosisporus
NR 026071.1-thermocarboxydovorans
NR 041182.1-glaucus
NR 041730.1-nodosus
NR 025869.1-eurythermus
NR 043346.1-aureoverticillatus
NR 027223.1-violaceolatus
NR 027222.1-coelescens
NR 041914.1-violaceoruber
NR 025250.1-humiferus
NR 025871.1-tendae
NR 043745.1-tritolerans
NR 025870.1-sampsonii
NR 026535.1-odorifer
NR 041065.1-albidoflavus
NR 043815.1-daghestanicus
NR 041115.1-violaceus
NR 025722.1-africanus
NR 041221.1-purpurascens
NR 043831.1-maganshii
NR 041101.1-fumanus
NR 041135.1-anandii
NR 025202.1-caelestis
NR 043835.1-pseudogriseolus
NR 041158.1-capillispiralis
NR 043815.1-cellulosae
NR 041157.1-carpinensis
NR 043508.1-atrovirens
NR 043840.1-variabilis
NR 044139.1-mutabilis
NR 044149.1-minutisclerotícus
NR 043818.1-geysiriensis
NR 043383.1-vinaceusdrappas
NR 041178.1-djakartensis
NR 043842.1-virens
NR 041122.1-calvus
NR 041193.1-asterosporus
0.01

(2) Characterization of the *Streptomyces lonarensis* strain NCL 716

Morphological characteristics

This strain grew optimally at 28^0C in alkaline medium and there was no growth in neutral medium. The optimal growth was obtained at 28^0C at pH 8.0 – 10.0 (Fig. 2.2) and there was no growth at pH less than 8.0, suggesting that the strain was a facultative alkalophile. The alkalophilic *Streptomyces lonarensis* strain NCL 716 is the sixth report of an alkalophilic *Streptomyces*, besides the four available reports of alkalophilic *Streptomyces* strains (Nakai *et al*, 1986; Moriyama *et al*, 2000; Li *et al*, 2005; Dastager *et al*, 2007; Chakraborty *et al*, 2009). The colony showed the typical dry powdery characteristic of an actinomycete colony. Aerial mycelial formation was extensive.

Fig 2.2: Growth of *Streptomyces lonarensis* strain NCL 716 in alkaline medium.

The scanning electron microscopy pictures of the culture grown on Potato Starch Agar medium are shown in Figs 2.3 - 2.5. Color of the mycelium is white. Spore chains are flexuous (wavy) and there is also a variation in the number of spores per chain (10-20 spores per chain). The sporophore can be described as Rectus-Flexibilis and the spore surface is smooth. Morphology of *Streptomyces lonarensis* strain NCL 716 resembles that of *S. diastaticus, S. alboniger,* three strains of *S. albus, S. nitrificans, S. rimosus* and *S. ruber* (Tresner *et al*, 1960).

Scanning electron microscopic observation of Streptomyces lonarensis strain NCL 716:

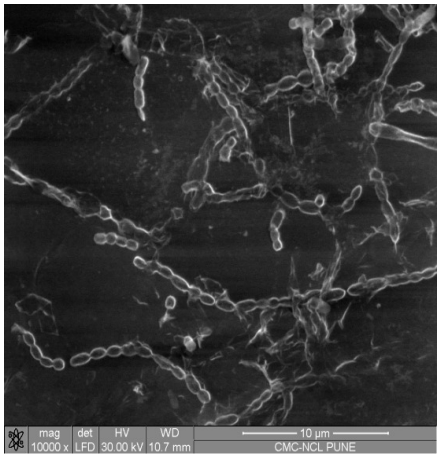


Fig 2.3: Culture from 21 day old plate

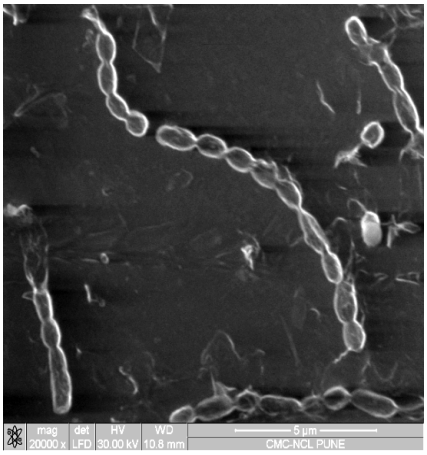


Fig 2.4: Culture from 21 day old plate

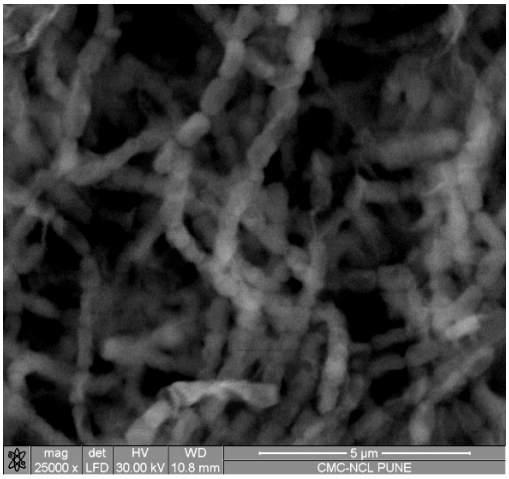


Fig. 2.5: Culture from a 21-day-old plate

Physiological and biochemical characteristics

The culture was grown on different media and observations were made on its growth, aerial and substrate mycelium, sporulation pattern and diffusible pigment (Table 2.1; Figures 2.6 – 2.13).

Table 2.1: Morphological and physiological characterization of *Streptomyces lonarensis* strain NCL 716.

Medium	Growth	Sporulation	Diffusible pigment	Colony	
				Aerial mycelium	Substrate mycelium
Czapeks agar	Poor	Poor	Absent	White	White
Glycerol-aspargine agar (ISP 5)	Good	Good	Absent	White	White
Inorganic salt-starch agar (ISP 4)	Good	Good	Absent	White	White
Nutrient agar	Good	Moderate	Absent	White	Grey-white
Oatmeal agar (ISP 3)	Very good	Very good	Dark brown	Grey	Grey-brown
PDA	Very good	Very good	Brown	White	Yellow-white
Tyrosine agar (ISP 7)	Poor	Poor	Reddish-brown	Light-pink	Brown
Yeast extract agar (ISP 2)	Very good	Very good	Brown	Light-brown	Off-white

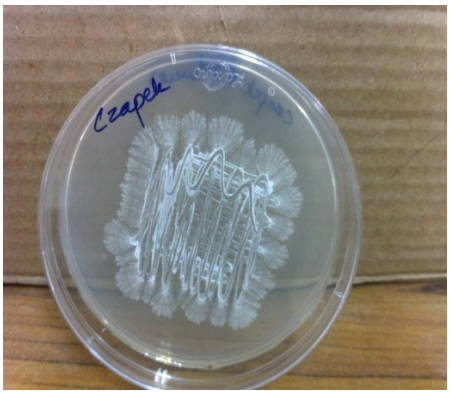


Fig 2.6. Czapeck agar

Fig 2.7. Glycerol-aspargine agar

Fig 2.8. Inorganic salt-starch agar

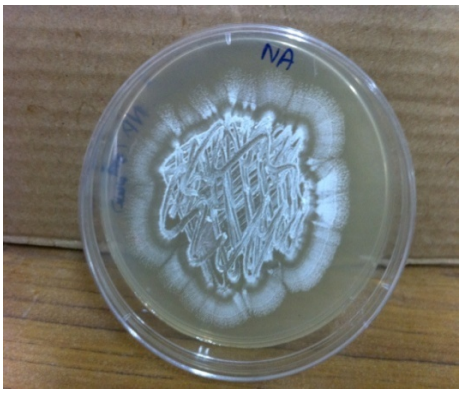


Fig 2.9. Nutrient agar

Fig 2.10. Oatmeal agar

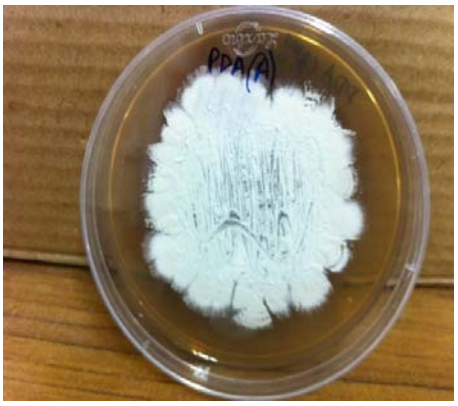


Fig 2.11. PDA

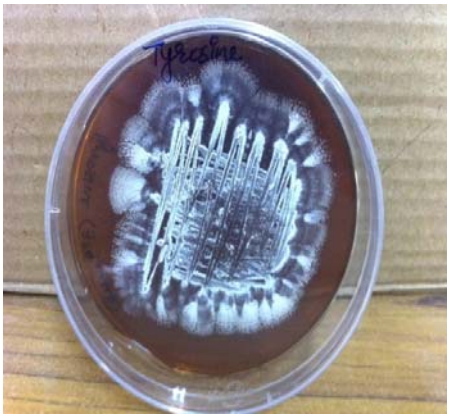


Fig 2.12. Tyrosine agar

Fig 2.13. Yeast extract agar

Effect of temperature on growth

No growth was observed in PSA plates, pH 9.0 incubated at 35°C, 40°C and 45°C. The optimum temperature of growth of the strain was 28°C - 30°C.

Effect of pH on growth

Good growth of the culture was observed in PSA plates at pH 8.0, 9.0, 10.0, and 11.0. Maximum growth was observed at pH 9.0. No growth was observed below pH 8.0, suggesting the *Streptomyces lonarensis* strain NCL 716 to be a facultative alkalophile.

Effect of NaCl on growth

The culture was grown on PSA plates containing varying amounts of NaCl (Figs 2.14 to 2.23). Maximum growth of *Streptomyces lonarensis* strain NCL 716 was observed at 1% NaCl concentration. As the concentration was increased, the growth decreased and beyond 6% NaCl concentration, no growth was observed. The *Streptomyces sp* NCL 716 grows in the absence of NaCl and hence is not a halophile. The strain can tolerate NaCl concentration of up to 6%.

Fig 2.14. 1% NaCl

Fig 2.15. 2% NaCl

Fig 2.16. 3% NaCl

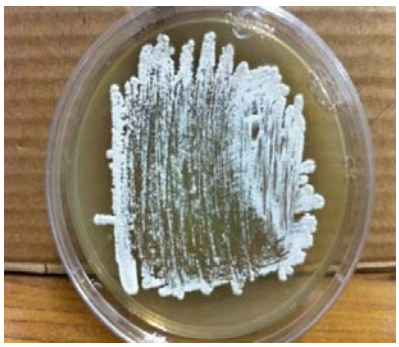


Fig 2.17. 4% NaCl

Fig 2.18. 5% NaCl

Fig 2.19. 6% NaCl

Fig 2.20. 7% NaCl

Fig 2.21. 8% NaCl

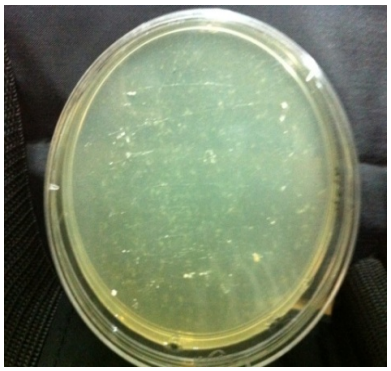


Fig 2.22. 9% NaCl

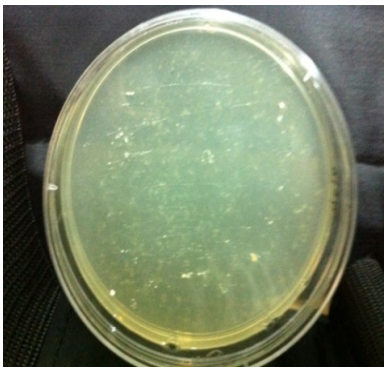


Fig 2.23. 10% NaCl

Utilization of carbon sources

The growth of the culture in the presence of different sugars as carbon sources was studied and the observations are summarized in Table 2.2.

Table 2.2. Utilization of various carbon sources by *Streptomyces lonarensis* strain NCL 716

Sugar\Hours	**growth**
Dextrose	++
Lactose	-
Maltose	++
Sucrose	-
Raffinose	+
Rhamnose	+
meso-Inositol	-
Xylose	++
Ribose	++
Arabinose	+
Adonitol	+
Fructose	++
Mannitol	++

[-: no growth, +: good growth, ++: luxurious growth]

Streptomyces lonarensis strain NCL 716 utilized carbon sources dextrose, maltose, xylose, ribose, fructose, mannitol very well. It could also utilize raffinose, rhamnose, arabinose, adonitol to some extent but could not utilize lactose, sucrose and meso-inositol.

Resistance to antibiotics

Zone of clearance was observed in plates containing ampicillin, chloramphenicol, gentamycin, novobiocin and refampicin within 24 h suggesting that the *Streptomyces lonarensis* strain NCL 716 was sensitive towards ampicillin (90 µg), chloramphenicol (30 µg), gentamycin sulphate (120 µg), novobiocin sulphate (90 µg) and rifampicin (10 µg) (Table 2.3). The maximum sensitivity of the strain was seen against rifampicin (10 µg). In plates containing other antibiotics, no zone of clearance was seen. The *Streptomyces lonarensis* strain NCL 716 was resistant to vancomycin hydrochloride, kanamycin sulphate, nystatin, nalidixic acid and tetracycline hydrochloride.

Table 2.3. Antibiotic resistance showed by *Streptomyces* strain NCL 716

Antibiotic (µg)	**Zone of clearance (diameter in mm)**
Ampicillin (90)	18
Chloramphenicol (30)	25
Gentamycin (120)	20
Novobiocin (90)	15
Rifampicin (10)	40

Antimicrobial activity:

The antimicrobial activity of the *Streptomyces lonarensis* strain NCL 716 was tested by using a cell-free extract of the strain against a few strains known to cause diseases in plants and humans, as described in Materials and Methods. Zones of clearance were seen in plates of *Bacillus subitilis* NCIM 2063, *Escherichia coli* NCIM

2065, *Pseudomonas aeruginosa* NCIM 2200, *Fusarium proliferatum* NCIM 1103, *Pseudomonas fluorescens* NCIM 2099 and *Staphylococcus aureus* NCIM 2079. The above-mentioned bacterial and fungal cultures are sensitive to the cell free extract of *Streptomyces lonarensis* strain NCL 716 strain suggesting that the cell-free extract contains some antimicrobial compound which inhibits the growth of these cultures. These cultures are known to cause the following diseases:

(1) *Bacillus subtilis* - Known to contaminate food and may result in food poisoning.
(2) *Candida albicans* – Known to cause oral and genital infections.
(3) *Curvularia lunata* – Fungal plant pathogen.
(4) *Escherichia coli* – Known to cause gastrointestinal infections, urinary infections and neonatal meningitis.
(5) *Fusarium proliferatum* – Fungal plant pathogen.
(6) *Pseudomonas aeruginosa* – Known to cause urinary tract infection, respiratory system infection, dermatitis, soft tissue infection, bacteremia, bone and joint infection, gastrointestinal infection and variety of systematic infections.
(7) *Pseudomonas fluorescens* – Known to cause milk spoilage by causing bitterness, casein breakdown, ropiness due to production of slime and coagulation of proteins. It is known to affect only compromised patients.
(8) *Staphylococcus aureus* – Known to cause skin infections like pimples, impetigo, boils, cellulites folliculitis, scalded skin syndrome and dangerous diseases such as pneumonia, meningitis, osteomycilitis, chest pain, toxic shock syndrome, bacteremia and sepsis.

Table 2.4 Antimicrobial activity by Streptomyces lonarensis strain NCL 716

Cultures	Zone of clearance
Bacterial Cultures	
Bacillus subitilis NCIM 2063	+++
Escherichia coli NCIM 2065	++
Pseudomonas aeruginosa NCIM 2200	+
Pseudomonas fluorescens NCIM 2099	+
Staphylococcus aureus NCIM 2079	+
Fungal Cultures	
Fusarium proliferatum NCIM 1103	+
Curvularia lunata	-
Candida albicans	-

Note - (**+**, weak inhibition; ++, good inhibition; +++, strong inhibition; -, no inhibition)

Hydrolytic reactions (degradation of starch) by the Streptomyces lonarensis* strain *NCL 716.

The *Streptomyce lonarensis* strain NCL 716 strain gave a distinct zone of clearance on starch agar medium when flooded with iodine solution in KI indicating the strain to be amylolytic (Fig. 2.24). The alkaline amylase produced by this strain is the fifth report, besides the four available reports of alkalophilic *Streptomyces* strains producing alkaline alpha-amylases (Nakai *et al*, 1986; Syed *et al*, 1999; Moriyama *et al*, 2000; Chakraborty *et al*, 2009).

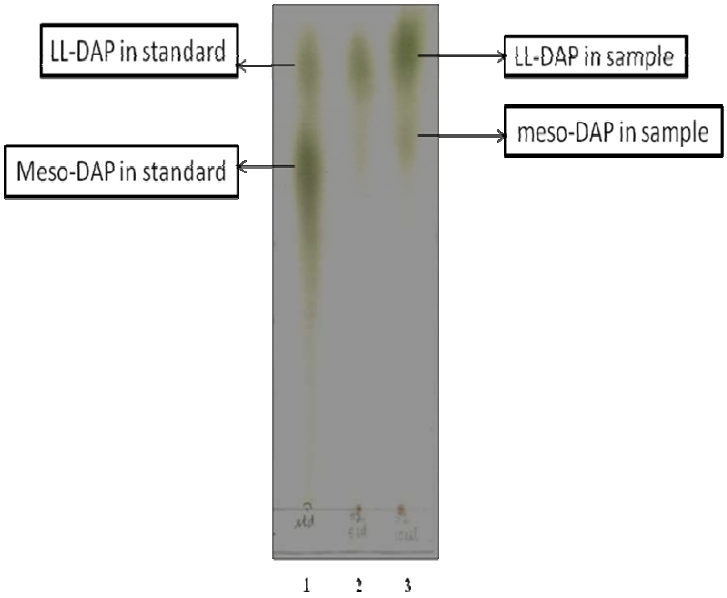
Fig 2.24. Amylolytic activity of *Streptomyces* strain NCL 716

(3) Chemotaxonomical characterisation of the *Streptomyces lonarensis* strain NCL 716

Estimation of the isomer of diaminopimelic acid in the cell wall

Paper chromatography of the cell wall hydrolysate showed a prominent spot corresponding to LL-A_2pm and a minor spot corresponding to meso-DL-A_2pm (Fig. 2.25). The cell wall of *Streptomyces* belongs to Cell Wall Type I which is characterised by the presence of LL-A_2pm and glycine and absence of arabinose and galactose (Lechevalier and Lechevalier, 1970). However, Wellington *et al* (1992) detected 1-16 % of meso-DAP in the whole cell hydrolysates of mycelium of some *Streptomyces* species (Table 2.5).

Fig. 2.25 Paper chromatogram showing the presence of LL-A_2pm



Lane 1: standard mixture of LL, meso and DL DAP, **Lane 2:** 5 µl of cell hydrolysate, **Lane 3:** 10 µl of cell hydrolysate.

Table 2.5 *Streptomyces* strains containing LL-DAP and Meso-DAP

Organism	Collection number	%DAP	
		LL	Meso
Streptomyces albus (S)	ISP 5313	95	5
Streptomyces albus (L)	ISP 5313	88	12
Streptomyces cyaneus (S)	JCM 4220	91	9
Streptomyces jlaveus (S)	A 2001	99	1
Streptomyces griseoruber (S)	JCM 4200	95	5
Streptomyces griseus (S)	ISP 5236	93	7
Streptomyces griseus (L)	ISP 5236	93	7
Streptomyces roseiscleroticus (S)	JCM 3104	85	15
Streptomyces purpureus (S)	JCM 3172	84	16

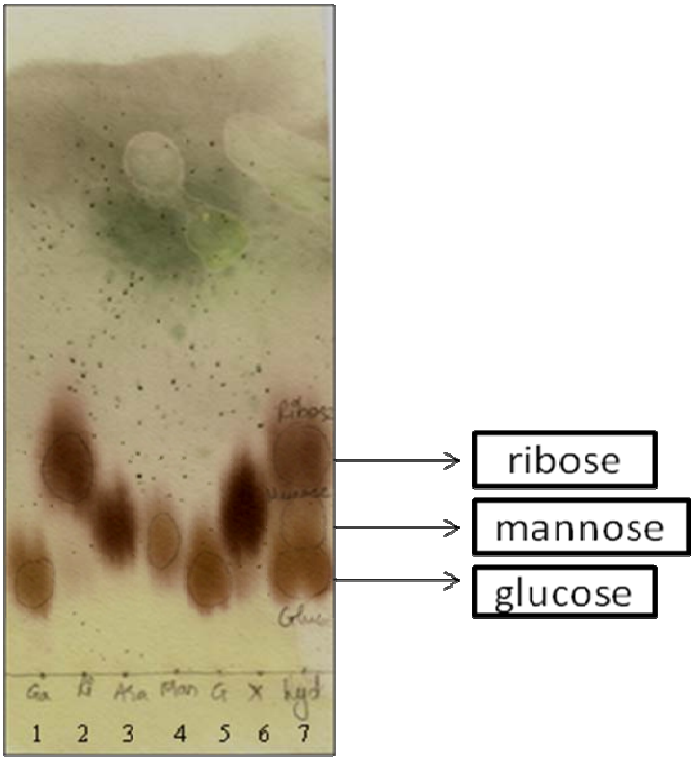
(S) – Submerged fermentation; (L) – liquid fermentation media

**** Data taken from*** Wellington *et al, 1992.*

Whole cell sugars

Cell wall type I lacks arabinose and galactose which are characteristic sugars of cell wall type IV. These two sugars are absent in the paper chromatogram of whole cell sugars of the *Streptomyces lonarensis* strain NCL 716. (Fig. 2.26), a characteristic of the cell wall of *Streptomyces* (Lechevalier and Lechevalier, 1970). Non-diagnostic sugars like glucose, mannose and ribose were seen in the paper chromatography of the hydrolysate.

Fig.2.26. Paper chromatogram of whole cell sugars of the *Streptomyces lonarensis* strain NCL 716.

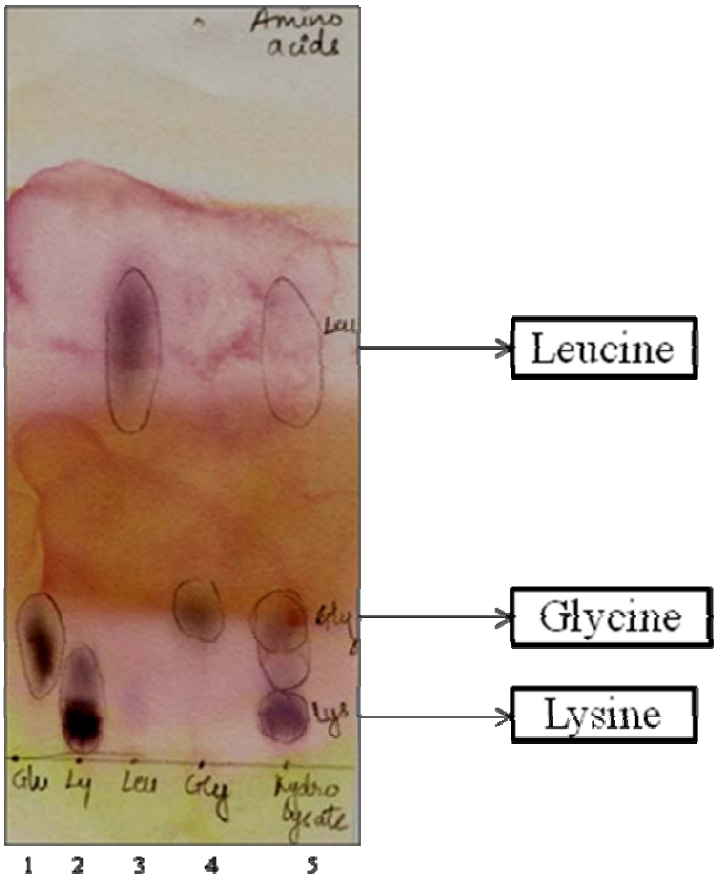


Lane 1: Galactose; Lane 2: Ribose; Lane 3: Arabinose; Lane 4: Mannose; Lane 5: Glucose; Lane 6: Xylose; Lane 7: cell hydrolysate

Cell wall amino acids

The paper chromatogram of the cell wall aminoacids shows a spot corresponding to glycine, the diagnostic amino acid present in cell wall type I (Lechevalier and Lechevalier, 1970) (Fig. 2.27). In addition, non-diagnostic amino-acids lysine and leucine were also seen in the paper chromatogram.

Fig.2.27. Paper chromatogram of the cell wall amino acids of *Streptomyces lonarensis* strain NCL 716.



Lane 1: Glutamic acid; Lane 2: Lysine; Lane 3: Leucine; Lane 4: Glycine; Lane 5: cell hydrolysate

Cell wall fatty acids

Nineteen fatty acids were identified by gas chromatography. The fatty acid composition of the cell wall of the *Streptomyces lonarensis* species NCL 716 strain is shown in Table 2.6.

Table 2.6: Fatty acids composition of the *Streptomyces* strain NCL 716

Fatty acids	Percent (%)
14:0 iso	0.65
15:0 iso	1.97
15:0 anteiso	5.09
15:0	1.53
16:1 iso H	7.22
16:1 cis 9	3.92
16:0 iso	46.87
16:0 iso 10 methyl	0.83
16:0 iso 9 methyl	3.15
16:0	4.10
17:1 anteiso	12.42
17:1 anteiso C	4.76
17:0 iso	1.29
18:0 iso	0.6
17:1 cis 9	1.49
17:0 cyclo	2.22
17:0	0.51
17:0 10 methyl	0.42
10:0 2OH	0.23
Unknown	0.60

The major branched fatty acids were 16:0 iso (46.87%) and 17:0 anteiso (12.42%). Amongst the saturated fatty acids, 16:0 iso (46.87%) was a major component followed by 17:0 anteiso (12.42%), 15:0 anteiso (5.09%), 16:0 (4.10%) and 16:0 9 Methyl (3.15%). Amongst the mono-unsaturated fatty acids, 16:1 iso-H (7.22%), 17:1 anteiso C (4.76%) and 16:1 cis 0 (3.92%) were present in significant amount. The detection of major amounts of saturated iso and anteiso branched fatty acids between 14 and 17 carbon atoms is in good agreement with the reports from other *Streptomyces* strains (Kaneda,1977; Lechevalier, 1977; Kroppenstedt and Kutzner,1978; Kroppenstedt, 1985; Saddler, 1987).

Ballio *et al* (1965) first reported the fatty acids composition of *S.aureofaciens, S. coelicolor, S.flavovirens, S. gelaticus, S.griseus* and *S.viridochromogenes*. Their results showed that in these *Streptomyces* species, the saturated fatty acids i.e C14, C15 and C 16 iso acids and the saturated C15 anteiso acid were most prominent. Subsequently, the fatty acids composition from a number of *Streptomyces* has been reported ie *S.hygroscopicus* (Grafe *et al*,1982; Hoischen *et al*, 1997) ; strains of *S .cyaneus* (Saddler *et al*, 1987); *S .fulvoviolaceus* (Geshava *et al*, 1998); *Streptomyces* Group A,B and C strains (Atalan *et al*, 2000); 38 alkali tolerant mesophilic strains of *Streptomyces* (Sahin *et al*, 2001); *S.sodophilus* (Li *et al*, 2005); *Streptomyces xiamenensis* (Xu *et al*, 2009); *Streptomyces* strain TN17, S. *lavendofoliae* NBRC 12882, *S.lilaceus* NBRC 13676 ^{T}, *S.gobitricini* NBRC 15419T (Smaoui *et al*, 2011). In *Streptomyces lonarensis* strain 716, the unsaturated and mono-unsaturated fatty acids accounts for almost 81.88 % and 17.39% respectively, of the total fatty acids. Such a membrane composition of highly unsaturated and branched fatty acids could contribute to a membrane that is functional at pH 9 or higher, but whose integrity is compromised at near neutral pH. This could explain why this strain does not grow at pH 7.0. This conclusion is drawn based on the observations by Clejan *et al* (1986) and Dunkley *et al* (1991) in their study on fatty acid composition of obligate and facultative strains of *Bacillus*.

The fatty acids composition of the *Streptomyces lonarensis* strain NCL 716 has been compared with that of the two other alkalophilic *Streptomyces* strains ie *S.gulbargensis* and *S.sodiiphilus* (Table 2.7*). S.sodiiphilus* grows at pH 7-12 (Li *et al*, 2005), while *S.gulbargensis* grows at pH 6-12 (Dastager *et al*, 2006). The *Streptomyces* strain NCL 716 does not grow at near neutral pH. This feature could be due to the relatively high percentage of 46.87% of iso C16:0 in this strain.

Table 2.7: Fatty acids composition of the reported alkalophilic *Streptomyces* strains

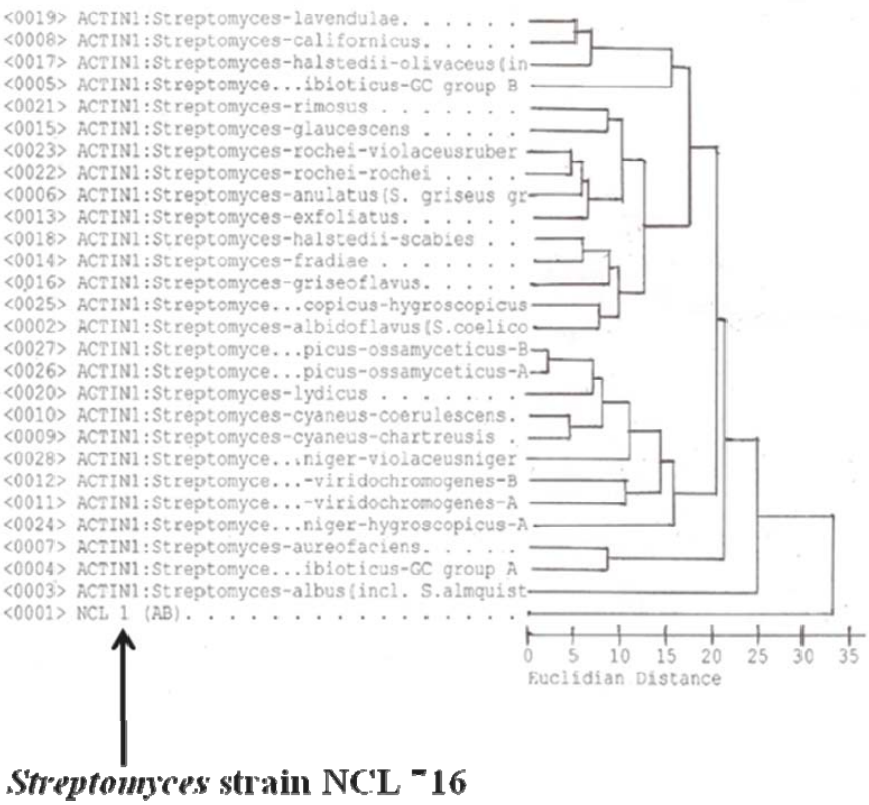
Fatty acid	*Streptomyces* strain NCL 716 (%)	*S.gulbargensis* (%)	*S.sodiiphilus* (%)
ante-iso C15:0	5.09	26	16.47
iso C16:0	46.87	20	31.32
iso C15:0	1.97	10	NA
ante-iso C17:0	12.42	9	13.30
C16:0	4.10	8	NA

*NA: Data not available

Fatty acid composition has been implicated to play an important role in maintaining the fluidity of membrane lipids which in turn is important for the growth of microorganisms (Clejan *et al*, 1986; Kaneda, 1977). The melting temperature of the fatty acids is an important factor which contributes to the fluidity of membrane lipids (Kaneda, 1977). The melting temperatures of the branched chain fatty acids are lower than the straight chain fatty acids with the same number of carbons. The anteiso fatty acids have melting temperatures that are almost 25-30°C lower than the normal series. It has been found that in *B.cereus*, *B.subtilis and B.stearothermophilus*, lowering the culture temperature results in an increase in the proportion of anteiso series, suggesting that the branched chain fatty acids in these organisms are responsible for maintaining the fluidity of the membranes at lower temperatures (Kaneda,1977). A similar explanation can be given in case of *Streptomyces lonarensis* strain 716 where a substantially high amount of branched fatty acids may allow this strain to grow at 28°C. Kaneda (1977) has also suggested that the branched chain fatty acids may function as efficient surface coating agents.

The fatty acids composition also has important implications in taxonomy and in establishing an evolutionary scheme for microorganisms (Kaneda, 1977). The relationship of the *Streptomyces lonarensis* strain NCL 716 with other *Streptomyces* strains available in the database of MIDI was depicted using a UPGMA dendrogram which was based on the coefficient of similarity generated by the Euclidean distance between pairs of strains (Fig. 2.28). The phylogenetic tree was developed using the program Sherlock (version 6.0B). At a Euclidean distance of about 0.33, the *Streptomyces lonarensis* strain NCL 716 separated from the other strains of the MIDI library.

Fig 2.28 Phylogenetic tree based on fatty acids analysis



rDNA analysis

The 16S rDNA nucleotide sequence of the *Streptomyces lonarensis* strain NCL 716 consisted of 1527 bp which covers almost the entire 16S rDNA. The neighbour-joining phylogenetic tree developed on the basis of the nearly complete 16S rDNA sequence of *Streptomyces lonarensis* strain NCL 716 (FJ 919811) and the first 100 Blast hits is shown in Figs 2.1 and 2.29.

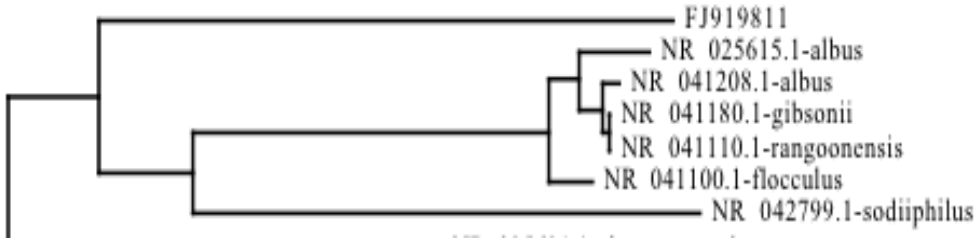


Fig 2.29. **Enlarged image from the phylogenic tree (Fig. 2.1) showing the cluster having the *Streptomyces lonarensis* strain NCL 716.**

This strain occupies a position in same cluster as *S. albus, S. flocculus and S. sodiiphilus.* Morphological characters of the *Streptomyces lonarensis* strain NCL 716 grown on ISP 2 medium (Table 2.8) and all other media (Table 2.9) were compared with the available data for the other three strains in the same cluster ie *Streptomyces albus,* (Shirling and Gottlieb, 1969), *Streptomyces flocculus* (Shirling and Gottlieb, 1969) *and Streptomyces sodiiphilus*, (Li *et al*, 2005) to find out if these strains shared common characters.

Table 2.8. Comparison of morphological characters on ISP 2 medium, of *Streptomyces lonarensis* strain NCL 716 with other closely related *Streptomyces* species grouped in the same cluster based on 16s rDNA analysis.

1) Streptomyces lonarensis strain NCl 716*, 2) Streptomyces albus, 3) Streptomyces flocculus, 4) Streptomyces sodiiphilus,*

Response characters are scored as positive, +; negative, -; variable, v.

The data are from the following references: Shirling and Gottlieb (1968a, 1968b, 1969 and 1972).

Morphological characters observed on ISP 2 medium	**1**	**2**	**3**	**4**
Spore chain	Flexous Chains	Spirales	Spirales	Straight to Retinaculiaperti
Spore surface	Smooth	Smooth	Smooth	Wrinkled Surface
Aerial mass colour	White	White/ Yellow color	White/ Yellow Color	Yellow-white
Formation of pigment	Brown black	No Pigment	No Pigment	No pigment
L-Arabinose	+	V	+	-
Raffinose	+	V	Trace	-
Rhamnose	+	-/trace	Trace	+
Sucrose	-	-/trace	+	-
D-xylose	+	+	+	-

Table 2.9. Comparison of the phenotypic characters of *Streptomyces lonarensis* strain NCL 716 with other closely related species that are grouped in the same cluster based on 16s rDNA analysis, on different media.

1) *Streptomyces lonarensis* strain NCl 716, **2)** *Streptomyces albus*, **3)** *Streptomyces flocculus*, **4)** *Streptomyces sodiiphilus.*

Medium		1	2	3	4
Glycerol Aspargine Agar (ISP 5)	**G**	+	+	+	+
	AM	White	White/ Yellow	White/ Yellow	White aerial mycelia
	SP	Absent	No pigment	Colorless/ Pale grayish yellow	No diffusible pigment
Inorganic Salts Starch Agar (ISP 4)	**G**	+	+	+	-
	AM	White	White/ yellow	White/ Yellow	White aerial mycelia
	SP	No pigment	No pigment	Colorless/ Pale grayish yellow	No diffusible pigment
Oatmeal Agar (ISP 3)	**G**	+	+	+	+
	AM	Grey	White color/ Yellow	White Color/ Yellow	White aerial mycelia
	SP	Dark Brown	No pigment	Colorless/ Pale grayish yellow	No diffusible pigment
Yeast extract malt extract agar (ISP 2)	**G**	+	+	+	+
	AM	Light Brown	White color/ yellow	White Color/ Yellow	White aerial mycelia
	SP	Brown	No pigment	Colorless/ Pale grayish yellow	No diffusible pigment

G - Growth, Aerial Mycelium – AM, Soluble Pigment – SP

The *Streptomyces lonarensis* strain NCL 716 differs from the other three strains in the following morphological characters :

(i) Its sporophore exhibits flexous chains.

(ii) It produces a brown pigment on ISP 2 medium and a dark brown pigment in ISP3 medium.

(iii) It produces a light brown mycelium in ISP2 medium and a grey mycelium on ISP3 medium.

The characteristics of the *Streptomyces lonarensis* strain NCL 716 was also compared with the available data of the three other alkalophilic strains ie *S.sodiiphilus* (Li *et al*, 2005), *S. gulbargiensis* (Dastager *et al*, 2006) and *Streptomyces sp.* D1 (Chakraborty *et al*, 2009) (Table 2.10).

Table 2.10. Comparison of characteristic features of *Streptomyces lonarensis* strain NCL 716 with *S. sodiiphilus, S. gulbargensis and Streptomyces sp. D1*.

Sr. No.	Characteristis feature	*Streptomyces* strain NCL 716	*S. sodiiphilus*	*S. gulbargensis*	*Streptomyces sp.* D1
1	pH of growth	8-12	7-12	6-12	8 – 11
2	Temperature of growth	28°C – 30°C	28°C – 30°C	28°C - 45°C. Optimum is 45°C	37°C – 55°C Optimum is 45°C
3.	Dependence of NaCl for growth	No	**YES (obligate dependence on NaCl)**	No	Moderately halophilic
4	Sporophore	straight to flexuous	straight to flexuous	straight	straight
5	Surface of the spore	Smooth	**NA**	smooth	warty
4	Production of melanoid pigment Medium ISP 6	ND	No	Yes	NA

	Medium ISP 7	Yes	No	Yes	
5	Utilisation of carbon source				
	- D-Glucose	Yes	Yes	Yes	NA
	- D-Arabinose	Yes	No	Yes	NA
	- D-Mannitol	Yes	No	Yes	NA
	- Fructose	Yes	No	Yes	NA
	- Raffinose	Yes	No	Yes	NA
	- D-Xylose	Yes	No	Yes	NA
	- L-Rhamnose	Yes	Yes	Yes	NA
	- Sucrose	No	No	**Yes**	NA
	- meso-Inositol	No	NA	Yes	NA
	Lactose	No	No	NA	NA
	Maltose	Yes	No	NA	NA
	Ribose	Yes	No	NA	NA
	Adonitol	Yes	NA	NA	NA
	D-galactose	ND	No	Yes	NA
6	Degradation of starch	Yes	No	Yes	Yes

ND : Not determined; NA : data not available.

Conclusions

The characteristic features of the actinomycete strain NCL 716 are s follows:

1. The actinomycete strain NCL 716 isolated from the meteorite salt water Lonar lake was identified to be a *Streptomyces* strain by rDNA analysis. Hence this isolate was named as *Streptomyces lonarensis* strain NCL 716 as it was isolated from the soil around Lonar lake. The rDNA sequence has been submitted to the GenBank with the accession number FJ919811.
2. Its cell wall was identified as Type I which is characteristic of the genus *Streptomyces*. However, trace amount of meso/DL DAP was also identified by paper chromatography.
3. The strain is a facultative alkalophile which grows at 28°C – 30°C and pH of 8-12. This is the sixth report of an alkalophilic *Streptomyces* strain.
4. It is halotolerant and can grow in the presence of upto 6% NaCl.
5. The colour of the aerial mycelium is white.
6. The sporophore can be described as Rectus-Flexibilis and the spore surface is smooth.
7. The strain utilizes glucose, maltose, raffinose, rhamnose, xylose, ribose, arabinose, adonitol, fructose and mannitol. The strain did not utilize lactose, sucrose and *meso*-inositol.
8. The strain produces melanoid pigment on ISP 7 medium.
9. The strain showed sensitivity towards rifampicin (10 µg).
10. The strain produces an alkaline amylase. This is the fifth report of an alkaline amylase from an alkalophilic *Streptomyces* strain.
11. This strain does not grow at neutral pH. This could be probably due to the high percentage of unsaturated and branched fatty acids along with the relatively high percentage of iso C16:0 (46.87%).

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Chapter 3

Optimization of fermentation for the production of the alkaline alpha-amylase using SVR and MJWA

Summary

Microbial amylases producing malto-oligosaccharides from starch have potential application in the food and pharmaceutical industries. Hence, identification of strains secreting such amylases and optimization of fermentation conditions to maximize enzyme yield is important. To achieve high product yields in a fermentation process, it is a prerequisite to design an optimal production medium and a set of optimal process operating conditions. The most widely used optimization method, single factor at a time, does not account for the combined effect of all the influential factors since other factors are maintained arbitrarily at a constant level. Further, this method is time consuming and requires a large number of experiments to determine the optimum levels of the production medium. Modeling and optimization of process parameters for the production of such enzymes is commonly done by support vector regression (SVR) technique and multicanonical jump walk annealing algorithm (MJWA), respectively. Multicanonical Jump Walk Annealing (MJWA) has been introduced for energy minimization in molecular modeling studies.

The *Streptomyces lonarensis* strain NCL 716 hydrolyses starch to produce a mixture of maltotriose (G3) and maltotetraose (G4) along with maltose (G2). The objective of the present work was to determine a cost-effective optimum media composition to obtain maximum yield of α-amylase from this strain. A SVR-based process model was developed for approximating the non-linear relationship between the fermentation operating variables and the α-amylase yield. The maximum amylase activity predicted by SVR was in good agreement with the experimental values at the optimized levels. The most influential factor was found to be starch while the least influential factor found was peptone. Peptone amount was kept constant throughout the fermentation. Peptone which is one of the expensive media components was used at a concentration of 1g/L, which made the optimum media composition cost effective. The optimum media composition obtained by this method was: Yeast Extract: 4.53g/L, Starch: 20.246g/L, K_2HPO_4: 0.0827%, $MgSO_4$: 0.15%, Peptone: 1g/L. A maximum enzyme activity of 297 U/ml which was achieved using the above approaches compares well with the activity of reported amylases producing maltooligosaccharides.

Introduction

The concept of functional foods i.e. foods or food ingredients that have a beneficial effect on human health is one of the leading trends in today's food industry. Interest in the use of malto-oligosaccharides with DP 3-10 as biopreservatives and functional foods has grown in recent years (Crittenden and Playne, 1996; Barreteau *et al*., 2006). The chemical manufacture of malto-oligosaccharides larger than maltotriose has been very difficult. However, the discovery of microbial enzymes that specifically act on starch substrate to produce specific malto-oligosaccharides has made it possible to produce syrups containing various malto-oligosaccharides (Okada and Nakakuki, 1992). There are very few amylases, which act on starch to specifically produce such malto-oligosaccharides (Tables 1.4 – 1.8). The focus of the present work is the alkalophilic *Streptomyces lonarensis* strain NCL 716 that produces an alkaline α-amylase, which specifically hydrolyzes starch to give maltotriose and maltotetraose as the predominant products in addition to maltose. The products maltotriose and maltotetraose, have properties that are compatible for use in the food industry (Barresi *et al*, 2003; Mussatto and Mancilha, 2007; (http://hayashibara-intl.com/food/food.htm).

Higher enzyme yields can be achieved by genetic alterations (Tanyildizi *et al*, 2005; Desai *et al*, 2006) or by formulation of an optimized media condition (Bhat *et al*, 2010). The conventional method of media optimization for enzyme production involves varying one factor at a time, which is time consuming and needs a lot of experimentation to determine the optimum levels of production medium. Further, enzyme production exhibits a nonlinear relationship with process variables. Hence, it is desirable first to develop a reliable nonlinear model based on optimum experimentation, followed by optimization using mathematical technique.

In recent years, support vector regression (SVR), a theory-based machine learning formalism is gaining popularity over conventional neural networks due to its many attractive features especially in cases where the development of phenomenological regression models that requires pre-specified data fitting function, becomes tedious or impractical (Gunn *et al.*, 1997; Vapnik, 1997, 1998). Detailed knowledge of the process phenomenology is not necessary for the model development and it can solely be constructed from the known process input–output data (training set).

Once an SVR-based process model is developed, a 'suitable' optimization technique that can work effectively with SVR can be applied for process optimization to obtain the optimal values of the process input variables that maximize or minimize a specified objective function. The conventional optimization technique which requires derivative of function cannot be used effectively; as the SVR models would not always be smooth especially in regions where in the input–output data (training set) used in model building is located sparsely.

Recently a new Stochastic Optimization Techniques, Multicanonical Jump Walk Annealing (MJWA) (Xu *et al*, 2000) has been introduced which combines the method of simulated annealing (SA) with the method of Multicanonical Ensembles (Berg *et al*, 1992) and assimilates their best features. This method has been found to be superior to simulated annealing (Datta *et al*, 2001).

The most frequently used operation in biotechnology is to improve the fermentation conditions for maximizing cell density, high level of desired metabolic product or enzyme levels in microbial system (Mukhopadhyay *et al*, 2008). This approach is time consuming and also ignores the combined interactions between physical as well as nutritional factors. Generally *Streptomyces* are a known source for the production of secondary metabolites and enzymes which have potential application in biotechnology.

The production of *α*-amylase by *Streptomyces sp.* ML12 was optimised using a combination of Plackett-Burman design, RSM (Response surface methodology) and central composite design (CCD). In this case, Plackett-Burman design was used to select the fermentation variables which were optimized by RSM. Five significant variables (rice bran and wheat bran - both are agricultural by-products), sodium chloride, magnesium sulphate and incubation period) were selected for the optimization via central composite design (CCD). The optimal features were rice bran (5.5 g/100 mL), wheat bran (5.3 g/100 mL), sodium chloride (2.8 g/100 mL), magnesium sulphate (1.4 g/100 mL) and 8 days of incubation period. Optimization of the medium with the above tested features increased the amylase yield by 4.4–fold. (Sivakumar *et al*, 2012). Optimization of *α*-amylase production by *Streptomyces erumpens* MTCC7314 under submerged fermentation was reported by Kar and Ray (2007), but it did not deal with

statistical optimization of the process. Production of α- amylase under solid state fermentation by *Streptomyces erumpens* MTCC 7317 was investigated using cassava fibrous residue. Response surface methodology (RSM) was used to evaluate the effect of the main variables, i.e., incubation period (60 h), moisture holding capacity (60%) and temperature (50°C) on enzyme production by applying a full factorial Central Composite Design. Varying the inoculum concentration (5-25%) of *S.erumpens* showed that 15% inoculum (v/w, 2.5 x 106 CFU/ml) was the optimum for α- amylase production. Beef extract was the most suitable nitrogen source for enzyme production (Kar and Ray, 2007). The statistical approach for *Streptomyces* sp can be used to optimize the production of industrially important enzymes and secondary metabolites. Response surface methodology was used to optimize the composition of medium for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A in shaker flask cultivation (Gao *et al*, 2009). The production of avermectin B1a was significantly influenced by corn starch and yeast extract by using the Plackett–Burman design. The steepest ascent method was used to access the optimal region of the medium composition, followed by an application of response surface. The analysis revealed that the optimum values of the tested variables were 149.57 g/l corn starch and 8.92 g/l yeast extract. A production of 5128 mg/l, which was in agreement with the prediction, was observed in verification experiment. In comparison to the production of original level (3528 mg/l), 1.45-fold increase had been obtained (Gao *et al*., 2009).

In the present work, an optimum media composition for the production of α-amylase by *Streptomyces lonarensis* NCL strain 716 has been achieved by a combination of the following strategies ie. (i) Placket–Burman (PB) has been used to select the most influential media components (ii) SVR technique has been developed using the influential process variables as model inputs and the α-amylase yield as the model output, and (iii) the input space of the SVR technique has been optimized using the MJWA formalism with a view of maximizing the α-amylase yield. A maximum enzyme activity of 297 U/ml achieved using the above approaches compares well with the activity of reported amylases producing malto-oligosaccharides (Sakano, 1983; Nakai, 1986; Nakada, 1990; Takasaki, 1991; Fogarty, 1994(a); Fogarty, 1994(b); Kim, 1995; Kobayashi, 1998; Dey, 2002)

Streptomyces lonarensis NCL strain 716 produces an alkaline α-amylase which hydrolyses starch to give G2 (Maltose), G3 (Maltotriose) and G4 (Maltotetraose). To date there are only four reports of alkaline amylases from this genus, two of which are patented. (Moriyama *et al*., 2000; Nakai *et al*., 1986; Chakraborty *et al*., 2009; Syed *et al*., 2009). In the genus *Streptomyces*, to date there is one report of a neutrophilic strain (Wako *et al*., 1979) and two reports of alkalophilic strains producing maltotriose (Syed *et al*, 2009; Chakraborty *et al*, 2009). There is only one report of an acidophilic strain producing maltotetraose (Cha *et al*, 1994).

Materials and Methods

Materials:

Malt extract, yeast extract, peptone, potato starch, agar, potayo starch. were procured from Hi- media chemicals, India. DNSA was from Sigma Chemicals, USA. Potassium sodium tartarate, di-Potassium hydrogen phosphate, Sodium carbonate, Magnesium sulphates were procured from Qualigens, India.

Methods

(A) Media for Amylase Production:

Potato Starch Agar (PSA)

A) For Working Plates:

Yeast extract	:	0.5 %
Peptone	:	0.5%
Potato starch (soluble)	:	1.0 %
$K_2H\ PO_4$	:	0.05%
$MgSO_4$	:	0.01%
Agar	:	2.4%
pH	:	10.0
Sodium carbonate	:	1.0 %

B) Media for Inoculum:

Media I

Yeast extract	:	0.5 %
Peptone	:	0.5%
Potato starch (soluble)	:	0.5 %
$K_2H\ PO_4$	:	0.05%
MgSO4	:	0.01%
pH	:	10.0
Sodium carbonate	:	1.0%

(C) Optimized media for fermentation

Potato starch (soluble)	:	2.0246 %
Yeast extract	:	0.453 %
$K_2H\ PO_4$	:	0.0827 %
$MgSO_4$	:	0.15 %
Peptone	:	1.0 %
pH	:	10.0
Sodium carbonate	:	1.0 %

Use of spore suspension in Tween 80

In the initial experiments, it was observed that each batch gave a different yield of amylase activity. This problem could be attributed to the difference in the number of viable spores that were being transferred to each inoculum and the fermentation flask in the different batches. Hence, there was a need to develop a standardized protocol to ensure that a nearly equal amount of spores was used in each batch. Therefore spore suspension was used instead of an agar piece from a 5 day old **PSA** (**P**otato **S**tarch **A**gar) plate. The spore suspension was prepared from a 7 day old **PSA** (**P**otato **S**tarch **A**gar) slant using 0.01 % Tween 80. The optical density of the spore suspension was

read at 600 nm. A fixed O.D value (of 0.5) was used, thereafter, in order to standardize the protocol. A fixed volume of this spore suspension was then used for media inoculation. The use of this method not only ensured reproducibility but also resulted in a rise of activity from 150 Units/mL to approximately 572 Units/mL **(these values of amylase yield were observed in the medium without any optimization)**. As per experimental data, maximum activity was attained between 108 h and 120 h of fermentation, using 1 mL of spore suspension in 0.01% Tween-80 and a 48 h old inoculum.

Plackett-Burman

Plackett and Burman (1946) developed a saturated fractional factorial design which has enabled the researcher to investigate accurately several factors simultaneously with no requirement to investigate all the possible combinations of factors (Durig and Fassihi, 1993). This design enables determination of the effect of variables with a minimum number of experiments. The disadvantage of this design is that it does not estimate the extent or type of interaction between variables (Motola and Agharkar, 1992). But, within the bounds of these limitations, the use of these screening procedures always results in a well designed, efficient experiment. The outcome of this design when combined with statistics attains significance. Thus, Plackett-Burman design has been recommended before formulation of compatibility studies.

Table 3.1. Estimation of most influential and lowest influential factor

Using the Plackett-Burman (PB) matrix, initial experiments were done. The most influential and lowest influential factor was determined. The matrix is as given below:

	Variables							
Trial	**Y.E**	**Peptone**	**Starch**	**K_2HPO_4**	**$MgSO_4$**	**Dummy**	**Dummy**	**Yield U/ml**
1	H	H	H	L	H	L	H	**148**
2	L	H	H	H	L	H	L	**200**
3	L	L	H	H	H	L	H	**25**
4	H	L	L	H	H	H	L	**60**
5	L	H	L	L	H	H	H	**111**
6	H	L	H	L	L	H	H	**250**
7	H	H	L	H	L	L	H	**122**
8	L	L	L	L	L	L	L	**41**

Based on these PB results, further RSM runs were designed. 25 runs were generated with each run having different composition. From the results obtained, only 22 runs were then trained using SVR and MJWA tools.

Strain and medium

Streptomyces lonarensis strain NCL 716 was isolated from the soil sample of Lonar lake (a meteorite lake) located in Buldhana district, Maharashtra State, India. The strain was maintained on neutral, alkaline PSA (Potato Starch Agar) medium at 28°C. The spores were also maintained on grass leaf and glycerol stocks were made and stored at -70˚C. The standard inoculum medium had the following composition (g/l): Yeast extract 5.0, peptone 5.0, starch 0.5%, K_2HPO_4 0.05%, $MgSO_4$ 0.01%, and

Na_2CO_3 (1%). The spore suspension was prepared from a 7 day old PSA slant using 0.01 % Tween 80. The optical density of the spore suspension was measured at 600 nm. A fixed O.D value (of 0.5) was used, thereafter, in order to standardize the protocol. A fixed volume of this spore suspension was added to the 50 ml inoculum media in a 250 ml conical flask, which was kept in an orbitary shaker at 28˚C and 160 rpm for 48 h. The pH of the medium after adding Na_2CO_3 was 10, which was the same as the production media. 5 ml of the above inoculum was transferred into 250 ml conical flasks containing 50 ml production medium. The production medium was composed of Yeast Extract 0.5%, Peptone 0.5%, Starch 3%, K_2HPO_4 0.1%, $MgSO_4$ 0.02% and Na_2CO_3 (1%). The flasks were then placed in an orbital shaker at 28˚C and 160 rpm for 120 h.

Enzyme analysis

After 120 h, the fermented broth was centrifuged at 10,000 rpm for 10 min at 28˚C. The supernatant was taken for estimation of enzyme activity since the α-amylase produced by this strain is extracellular. The amylase activity of the culture was estimated using soluble starch as the substrate by the dinitrosalicylic acid method as described by Bernfeld (1955). The DNS assay procedure was routinely used to evaluate enzyme activities. The reaction mixture (1 ml) contained 0.5 ml. of suitably diluted enzyme in glycine-NaOH buffer (50 mM pH- 9.0) and 0.5 ml of 1% starch solution. The reaction mixture was incubated at 45^0C for 30 min. and the reaction was terminated by the addition of 1 ml DNS. The tubes were heated in a boiling water bath and the colour intensity was read at 540 nm after dilution with 10 ml of distilled water (Bernfeld, 1955). A standard glucose curve was used for calculating enzyme activities. One unit of amylase activity is defined as the amount of enzyme required for producing 0.5 mg glucose or equivalent in 30 min under the assay conditions.

Support Vector Regression

Contrast to conventional neural networks, SVR formulation embodies the Structural Risk Minimisation (SRM) principle instead of traditional Empirical Risk Minimisation (ERM) principle which has been proved to be effective and equips SVR with greater potential to generalize (Basak, 2007). Basically SVM was developed for

classification, later Vapnik *et al*, 1997 proposed SVM for Regression (SVR). SVR is the most common application form of SVMs (Smola, 1998). An overview of the basic ideas underlying support vector machines and function estimation has been well described (Burges, 1998; Smola, 1998; Vapnik, 1998). The most important concept of SVM is the application of minimizing an upper bound to the generalization error instead of minimizing the training error based on this principle (Lee, 1993; Burges, 1998; Jain, 2007). SVMs achieved an optimum networks structure. In addition, the SVMs will be equivalent to solving a linear constrained quadratic programming problem so that the solution of SVMs is always unique and globally optimal. The basic idea in support vector regression (SVR) is to map the input data x into higher dimensional feature space F via a nonlinear mapping $\phi(x)$ and then a linear regression problem is obtained and solved in this feature space. Therefore, the regression approximation addresses the problem of estimating a function based on a given data set $G = \{(x_i, d_i)\}_{i=1}^{l}$ (x_i is input vector, d_i is the desired value). In SVM method, the regression function is approximated by the following function:

$$y = \sum_{i=1}^{l} w_i \phi_i(x) + b \qquad (1)$$

where $\{\phi_i(x)\}_{i=1}^{l}$ are the features of inputs, $\{w_i\}_{i=1}^{l}$ and b are coefficients. The coefficients are estimated by minimizing the regularized risk function. Another SVR variable, deviation, ξ and positive constant, C have to be chosen by the user and the optimal values are usually data and problem dependent. All computations are carried on via kernel function in the input space. The most used kernel functions are Gaussian RBF with a width of σ: $K(x, y) = \exp(-0.5\|x - x\|^2 / \sigma^2$ and the polynomial kernel with an order of d and constants a_1 and a_2: $K(x, y) = (a_1 x_i^T x + a_2)d$.

To determine extent of correlation between input and output data by the SVR, the root mean square (RMSE) error is defined as:

$$RMSE = \frac{\sqrt{\Sigma_{i=1}^{n} (y_i - \hat{y}_i)^2}}{n} \qquad (2)$$

where y_i is the measurements of the i^{th} output variable, and $\hat{y}_i$ is its estimate obtained from SVR model, and *n* is the total number of data points.

Multicanonical Jump Walk Annealing (MJWA)

MJWA combines the method of SA with the method of Multicanonical Ensembles. Simulated annealing is a stochastic technique for finding near global, optimum solutions and draws inspiration from the thermodynamic process of cooling (annealing) of molten metals to attain the lowest free energy state. The cooling phenomenon is simulated by controlling a temperature-like parameter introduced with the concept of the Boltzmann probability distribution. According to the Boltzmann probability distribution a system in thermal equilibrium at a temperature T has its energy distributed probability according to $P(E) = \exp(-E/kT)$, where k is the Boltzmann constant. At higher temperature SA can cross barriers in the function landscape but when temp anneals to low value sufficient excitation is not available to do so, thus SA can get trapped in local optima for several multimodal functions, and also acceptable solution may not be obtained for poor initial guesses. These drawbacks can be easily overcome by attaching method of multi canonical ensembles. In the multicanonical ensemble the configurations are sampled with the probability (or weight factor), $\rho_{mu}(E)$ inversely proportional to the density of states of the corresponding energy,

$$\Omega(E) \text{ , i.e. } \rho_{mu}(E) = \prec \frac{1}{\Omega(E)} \tag{3}$$

With this weight factor, the energies are sampled with a flat distribution

$$P(E) \prec \Omega(E)\rho_{mu}(E) = \Omega(E)\frac{1}{\Omega(E)} = 1 \tag{4}$$

Therefore, both the high-energy barriers and the low energy minima are explored with sufficient frequency. The density of states $\Omega(E)$ is not known a priori, and has to be numerically estimated via iteration (Lee, 1993; Hansmann, 1997), its calculation is main driving force for algorithm (Berg, 1992).

Since the probability that a certain energy E is sampled, is proportional to the weight factor $\rho(E)$ used in the multiplied by the density of energy states $\Omega(E)$, the energy histogram H (E) that gives the frequency of occurrence of the particular range of energies in the sampling should satisfy

$$H(E) \prec \Omega(E)\rho(E) \ldots\ldots\ldots \quad (5)$$

By using this microcanonical entropy S (E) can be found out. The entropy is used in the next iteration to calculate the weight factor, $\rho_{mu}(E)$. Once weight factor is known density of energy state can be estimate easily from equation (3) If the lowest and highest energies sampled in the simulation are *Emin* and *Emax* , respectively, we can estimate the density of states $\Omega(E)$ for *Emin*<*E*<*Emax* from equation (5) . Defining the entropy function

$$S(E) \prec \ln(\Omega(E)) \quad (6)$$

We have,

$$S(E) = \ln(\Omega(E)) = \ln(H(E)) - \ln(\rho(E)) \quad (7)$$

Where, $Emin \leq E \leq Emax$

MJWA shuttles back and forth between the canonical and multicanonical ensembles (hence, the name 'Jump Walk') and search the high and low energy ends of a function with equal probability and thereby locate the deepest local.

Problem formulation

After developing SVR-based process model with good prediction accuracy, a MJWA algorithm can be applied to optimize its input space (x) representing process variables, for maximizing the process performance. The prime objective of MJWA-based process optimization was to obtain the optimal values of influential fermentation operating

variables in order to maximize α-amylase yield. Since the SVR model represents the non-linear relationship between the four fermentation variables and α-amylase concentration, the same can be used to define the corresponding single objective optimization problem as stated below:

Maximize $y = f(x, w)\ x_i^L \leq x_i \leq x_i^U,\ i = 1, 2, ..., N$ (8)

Where f (.) represents the objective function (SVR model); y refers to the α-amylase yield; the decision vector, x, of dimension L (L = 4) denotes the fermentation operating conditions and x_i^L and x_i^U respectively, represent the lower and upper bounds on x_i.

Results and discussion

Selection of influential media component

Five parameters namely yeast extract, starch, K_2HPO_4, $MgSO_4$, peptone and time, were found to influence the production of amylase from starch. The initial screening design was done using Plackett- Burman before stepping on to optimization. The media component peptone (0.5 g in 1000 ml) which is commercially very expensive and functions as a nitrogen source, was found to be insignificant or the lowest influential factor in alpha amylase production by Plackett Burman method.

The process data for SVR-based modelling was generated by carrying out a number of fermentation runs by varying the input conditions as shown in Table 3.2. For SVR-based modeling, the process data comprising 22 patterns (example set) each representing a pair of model inputs (fermentation conditions) and a single output (amylase concentration), was partitioned into a training set (17 patterns) and a test set (5 patterns).

Table 3.2: Fermentation data used in SVR modelling

Sr. No.	Yeast Extract	Starch	K_2HPO_4	$MgSO_4$	Time	Enzyme Activity (SVM)	Enzyme Activity (Experimental)
	(g)	(g)	(%)	(%)	(h)	(U/ml)	(U/ml)
1	0.125	1.9375	0.08125	0.008125	120	96.40646	104
2	0.125	1.9375	0.19375	0.008125	120	63.50177	77
3	0.125	1.9375	0.08125	0.01935	120	156.09880	158
4	0.275	1.9375	0.08125	0.01935	120	102.09673	99
5	0.35	1.375	0.1375	0.01375	120	43.89228	46
6	0.2	2.5	0.1375	0.01375	120	90.68279	105
7	0.2	1.375	0.1375	0.025	120	53.89387	57
8	0.2	1.375	0.1375	0.01375	120	153.47267	159
9	0.275	0.8125	0.08125	0.01935	120	297.00000	297
10	0.275	0.8125	0.08125	0.01935	96	225.50789	211
11	0.125	1.9375	0.08125	0.01935	96	99.14818	106
12	0.2	1.375	0.1375	0.01375	96	127.41804	140
13	0.125	0.8125	0.08125	0.008125	72	20.00000	23
14	0.125	0.8125	0.19375	0.008125	72	20.00000	20
15	0.275	0.8125	0.08125	0.01935	72	123.78311	137
16	0.125	0.8125	0.19375	0.01935	72	20.00000	20
17	0.275	0.8125	0.19375	0.01935	72	20.00000	21
18	0.125	0.8125	0.19375	0.01935	120	65.92768	59
19	0.2	2.5	0.1375	0.01375	96	86.58274	89
20	0.2	1.375	0.25	0.01375	96	63.96952	73
21	0.218	1.024	8.25E-02	1.91E-02	120	291.14376	291
22	0.233	1.056	9.26E-02	2.04E-02	120	275.99762	275

During MJWA-implementation, the search for the optimal solutions was restricted to the following ranges of the four process operating variables: (1) Yeast extract concentration (g/l) (x_1): [0.125,0.35], (2) Starch concentration (g/l) (x_2): [0.8125,2.5], (3) K_2HPO_4 concentration / percentage (g/l) (x3): [0.08125,0.19375], and (4) MgSO4 percentage (x4): [0.008125, 0.025]. These ranges are the same over which the process operating variables were varied for collecting the experimental data listed in Table 3.1. After building SVR model for amylase production, MJWA-based optimization simulations were repeated by using a different randomly initialized population of the four input variables each time.

Accordingly, the five best solutions obtained after conducting numerous MJWA trials are given in Table 3.3. It was also observed that despite beginning the search in a different search space, the MJWA converged to similar optimal solutions indicating that the MJWA has indeed captured the solution corresponding to the tallest local or global maximum on the objective function surface. From the MJWA-optimized solutions listed in Table 2, it was seen that the best set of fermentation conditions (solution II) was expected to result in the α-amylase yield of 297 U/ml.

Table 3.3: Comparison of predicted enzyme activity by SVR technique to experimental enzyme activity under optimized levels:

Optimal concentration (Independent Variables)						Enzyme Activity	
Sr. No.	Yeast Extract	Starch	K_2HPO_4	$MgSO_4$	Time	SVM/ Predicted	Experimental
	(g)	(g)	(%)	(%)	h	(U/ml)	(U/ml)
1	0.225	1.09	8.78E-02	1.92E-02	120	288	286
2	0.2265	1.0123	8.14E-02	1.91E-02	120	297	297
3	0.228	0.824	8.79E-02	1.95E-02	120	291	290
4	0.227	1.116	8.30E-02	1.91E-02	120	285	288
5	0.226	1.103	8.19E-02	1.92E-02	120	287	287

MJWA was run for total 4000 iterations. This result was verified by carrying out a fermentation run using the MJWA-specified optimum conditions. The α-amylase yield obtained in the verification experiment was 297 U/ml, which was in agreement with the MJWA-optimized α-amylase yield of **297 U/ml.**

It was thus observed that the usage of a combination of statistical and SVR-based modelling and optimization methods could improve the enzyme activity significantly.

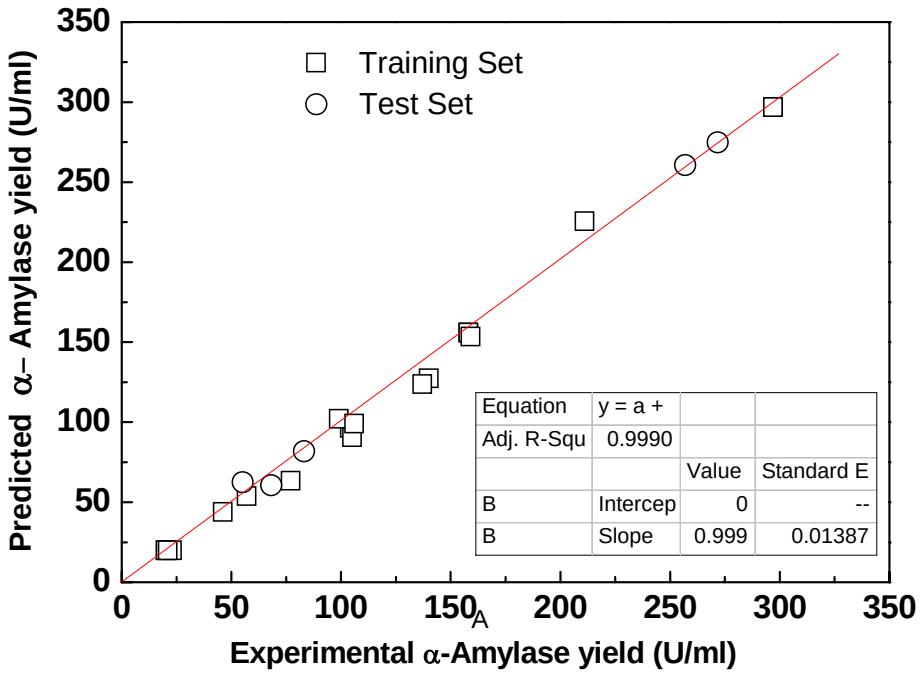
The maximum amylase activity predicted by SVR is in good agreement with the experimental values (297 U/ml) at the optimized levels .The amylase activity from this alkalophilic *Streptomyces lonarensis* strain NCL 716 has been bench marked with the amylases producing specific maltooligosaccharides from other *Bacillus* and *Pseudomonas* species (Table 3.4). From this table, it is seen that the NCL strain is promising and would have a potential in the food industry for the production of malto-oligosaccharides. The approach presented in this chapter is sufficiently general and thus can also be employed for modeling and optimization of other bioprocesses.

Table 3.6. Amylase activity produced by microorganisms

Organism	Enzyme produced (IU/ml)	Reference
Bacillus circulans GRS 313	98	Dey *et al*. (2002)
Bacillus sp. GM 8901	0.75	Kim *et al*. (1995)
Bacillus sp. MG-4	0.88	Takasaki *et al* (1991)
Pseudomonas sp. IMD353	29	Fogarty *et al* (1994 a and b)
Pseudomonas sp. MS300	88	Kobayashi *et al* (1998)
Pseudomonas sp.	6.5	Sakano *et al*. (1983)

Pseudomonas stutzeri MO-19	80	Nakada *et al* (1990)
Streptomyces sp.	0.85	Nakai *et al*. (1986)
Streptomyces lonarensis strain NCL 716	29.7	Present work.

Fig 3.1 Parity plot of alpha amylase yield.



Comparison of the network-predicted and desired values of the α-amylase yield is depicted in Fig.3.1. As can be noticed, the SVR-MJWA based model had fitted the experimental data with an excellent accuracy.

Hence the use of SVR-MJWA has proved to be very effective and can be used to design a cost effective media and to formulate an optimum composition for maximum α-amylase production.

Conclusions:

1. The optimum media composition obtained by this method is:
   Yeast Extract: 4.53g/l, Starch: 20.246g/l, K_2HPO_4: 0.0827%, $MgSO_4$: 0.15%, Peptone: 1g/l.
2. A combination of Plackett-Burman matrix and SVR-MJWA has yielded an optimized fermentation media which was much cost effective than the conventional method of media designing.
3. This is the first attempt wherein the Plackett-Burman process and SVR-MJWA tool are used in combination to design an optimized media for alkaline α-amylase production from an alklaophilic *Streptomyces*.

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Chapter 4

Purification and characterization of alkaline alpha amylase from *Streptomyces* strain NCL 716

Summary

Production of α-amylase from *Streptomyces lonarensis* NCL strain 716 *(*Amy Strplo 716*)* was carried out in shake flasks by growing the organism in a medium containing potato starch at pH 9.0 for 120 h. The enzyme was purified to homogeneity using the following steps: (i) Ammonium sulphate precipitation (90% saturation) and (ii) Preparative PAGE (5%). The enzyme showed maximum activity at pH 9.0 and at 45°C. The molecular mass of the Amy Strplo 716 on SDS-PAGE was estimated as 37,000 Da. The K_m and V_{max} values with starch, amylose, amylopectin were 2.0 mg ml^{-1}, 1.42 mg ml^{-1}, 2.0 mg ml^{-1} and 2.43 U, 2.42 U, 2.27 U respectively. The enzyme hydrolyzes starch to produce maltose, maltotriose and maltotetraose as observed by HPLC analysis. The pH activity profile of the enzyme revealed the participation of two ionizable groups with a pKa of pH 7.7 and 8.7 indicating the possible involvement of histidine and/or cysteine and arginine group at the active site, respectively. The secondary structure elements were estimated to be α-helix-12%, β-sheet-33.6%, turns-21.6% and unordered-32.4%. The fluorescence emission maximum of the protein with the excitation 295 nm was at 357 nm indicating tryptophans to be present in the polar environment. The decay curves obtained from the lifetime measurement of intrinsic fluorescence of Amy Strplo 716 revealed two life times, τ1 (1.5314 ns) with 94.14 % contribution and τ2 (5.9697 ns) with 5.86 % contribution, indicating the presence of two fluorophores differentially emitting the energy. The energy of activation of Amy Strplo 716 was estimated to be 18.36 KJ mol^{-1}.

Introduction:

Amylolytic enzymes play an important role in the degradation of starch (Sather, 1969). As compared to most amylases which exhibit pH optima in the acid or neutral range, the number of amylases with pH optima in the alkaline range is limited (Boyer *et al*, 1972) (Table 1.2). In the genus *Streptomyces*, there are only four reports of alkaline amylases (Moriyama *et al*, 2000; Nakai *et al*, 1986; Chakraborty *et al*, 2009; Syed *et al*, 2009). Amylases that hydrolyse starch to give specific oligosaccharide have been characterised from a number of organisms (Tables 1.4 – 1.8). In the genus *Streptomyces*, to date there is one report of a neutrophilic strain (Wako *et al*, 1979) and two reports of alkalophilic strains producing maltotriose (Syed *et al*, 2009; Chakraborty *et al*, 2009). There is only one report of an acidophilic strain producing maltotetraose (Cha *et al*, 1994).

Some of the methods that are used to characterise proteins are briefly described below:

Chemical Modification

The chemical modifications of reactive amino acid chains in proteins have immense applications in modern science. The chemical modification studies can be used for structure - function applications involving residues in enzyme active sites. Despite the increasing power of mutagenesis methods, it is advisable to use both the methods in combinations. Chemical modification studies can be used to identify likely residues responsible in a particular biological function and mutagenesis studies can be used to confirm these initial observations.

Generally, oxidation, reduction and nucleophilic or electrophilic substitutions are the most common chemical modifications that target the most reactive amino acid side chains. The microenvironments of the individual reactive side chains differ widely, which is a limitation affecting reactivity in some side chains. (Speicher, 1996).

Fluorescence measurements

Fluorescence spectroscopy is widely used to study peptides and proteins. The aromatic amino acids; viz tryptophan, tyrosine, and phenylalanine act as intrinsic fluorescent probes for studying protein conformation, dynamics, and intermolecular interactions. Of the three, tryptophan is the most popular probe. Tryptophan occurs as one or a few residues in most proteins and biologically active peptides.

Biophysical studies of protein

It is of great importance to study the folding, stability and aggregation of proteins. Biophysical studies can also be addressed as bridge between biology and physics. The chemical reactions in the body are performed by proteins. Biophysical studies help in understanding the concept of functioning of proteins. The response to drug differs due to variations in structure and/or proteins. This property can be used in drug designing, diagnosis and disease control.

Biophysical studies help in proper understanding of protein folding and provide tools for treating diseases.

Protein Folding

Protein folding is the process in which a linear polypeptide chain transforms into three-dimensional functional structure. A defined mechanism that governs folding remains a significant point in biophysics and molecular biology. The linear information of genetic message is translated into a three dimensional and functional structure of protein and this makes protein folding a topic of basic interest (Oas and Kim, 1988).

A specific three-dimensional structure makes the protein biologically active, which is common amongst all proteins. The genetic information is responsible for the primary structure of the protein and the linear sequence of amino acids in the polypeptide backbone. There is a spontaneous refolding exhibited by most of the protein in vitro after being completely unfolded. The three-dimensional structure must be determined by the primary structure. The reason behind this happening is yet unknown and this is 'the protein folding problem' (Creighton, 1990).

The protein folding problem is comprised of (i) kinetic process or pathway of the protein in its native and biologically-active folded conformation, (ii) the physical basis of the stability of folded conformations, (iii) the reason behind the amino acid sequence determine one particular folding process resulting in three-dimensional structure.

Protein folding intermediates

A number of equilibrium and kinetic studies lead to structural characterization of folding / unfolding intermediates. These intermediates are prerequisite and in a way contribute to solve the folding problem. Partially folded states are characterized at equilibrium under mildly denaturing conditions, such as by altering pH, addition of salts

and alcohols, chemical denaturants such as urea and guanidine hydrochloride or by changing temperature and pressure. The protein exhibits molten-globule like intermediate with rearranged secondary and tertiary structures and exposed hydrophobic amino acids on the surface. (Kuwajima,1989).

Significance of protein folding

Proteins are the most important molecules found in living organisms. They are required and are used to support the skeleton, control senses, move muscles, digest food, defence

against infections and process emotions. Christian Anfinsen in the early 1960's, showed that the proteins actually coil around themselves. If proteins become unfolded, they fold back into proper shape of their own accord without any requirement of a shaper or folder. There is a possibility wherein the protein will fold incorrectly. This explains the theory to protein folding diseases (King, 1993; Thomasson, 1996).

Materials and methods

Production of the α-amylase from *Streptomyces lonarensis* strain 716

The seven days old *Streptomyces species* strain NCL 716 grown on the Potato Starch Agar (PSA) slant were inoculated in a standard inoculum medium with the following composition: Yeast extract 0.5%, peptone 0.5%, starch 0.5%, K_2HPO_4 0.05%, $MgSO_4$ 0.01%, and Na_2CO_3 (1%). The pH of the medium was between 9.0-10.0. The spore suspension was prepared from a 7 day old Potato Starch Agar slant using 0.01 % Tween 80. The optical density of the spore suspension was measured at 600 nm. A fixed O.D value (of 0.5) was used, thereafter. A fixed volume of this spore suspension was added to the 50 mL inoculum media in a 250 ml conical flask, which was kept in an orbitary shaker at 28C and 160 rpm for 48 h. The pH of the medium after adding Na_2CO_3 was 10, which was same as that of production media. 5 ml of the above inoculum was transferred into 250 ml conical flasks containing 50 ml production medium. The production medium composition was Yeast Extract (0.453%), Starch (2.0246%), $MgSO_4$ (0.0191%), K_2HPO_4 (0.0814%) and Peptone (0.1%). After 120 h the fermented broth was centrifuged at 10,000 rpm for 15 min in cold. The supernatant was

used as the source of enzyme since the Amy Strplo 716 produced by this strain is extracellular.

Enzyme assay

The amylase activity in the culture broth was estimated using soluble starch as the substrate by the dinitrosalicylic acid method as described by Bernfeld, 1955. The DNS assay procedure was routinely used to evaluate enzyme activities. The reaction mixture (1 ml) contained 0.5 ml. of suitably diluted enzyme in glycine NaOH buffer (50 mM pH- 9.0) and 0.5ml of 1% starch solution. The reaction mixture was incubated at 45^0C for 30 minutes and the reaction was terminated by the addition of 1ml of DNSA. The tubes were heated in a boiling water bath and the colour intensity was read at 540 nm after dilution of the solution with 10 ml of distilled water (Bernfeld, 1955). A standard maltose curve was used for calculating enzyme activities. One International unit (IU) of amylase activity was defined as the amount of enzyme required to produce one micromole of maltose or equivalent in one min under the assay conditions.

Purification of the Amy Strplo 716 enzyme

The culture broth was concentrated by salt precipitation using ammonium sulphate till 90% saturation by gently stirring and adding salt at 4°C. Precipitate was collected by centrifugation at 10,000 rpm for 15 minutes, dissolved in Tris-HCl 10 mM, pH 7.6 and dialyzed extensively against the same buffer.

5 mg of the above sample was subjected to Preparative polyacrylamide gel electrophoresis (PAGE 5 % acrylamide). The run took 40-44 h. The enzyme on the gel was located by cutting a vertical strip which was further cut into 1 cm pieces. Pieces were crushed, suspended in the 50 mM glycine-NaOH buffer, pH 9.0. To this 2% starch substrate was added and incubated at 45°C for 5 minutes. The amount of glucose liberated was estimated by adding DNSA to the assay mixture and boiling it for 2 minutes. The portion of the gel corresponding to the piece showing Amy Strplo 716 activity was cut finely and crushed with glass rod and 20 ml of Tris-HCl pH 7.6 was added to it. The suspension was filtered and the filtrate was dialyzed against Tris-HCl, pH 7.6.

2 µg of enzyme was used for all the experiments unless or otherwise stated.

Protein concentration estimation

Protein concentration was determined according to the method of Lowry (Lowry and Rosebrough, 1951) using bovine serum albumin as standard.

Kinetics of Amy Strplo 716 with different substrates

K_m and V_{max} of Amy Strplo 716 for different substrates soluble starch, amylose and amylopectin were determined by Lineweaver-Burk plot method at 45°C and pH 9.0.by varying the substrate concentrations. 2µg protein was used for each assay. The activity of the enzyme was expressed as units/µmol of enzyme/min.

Determination of optimum pH and pH stability of Amy Strplo 716

Amy Strplo 716 assay was performed at different pH (4.0 to 11.0) at 45°C. The following buffers (50 mM) were used for these studies: Glycine-HCl for pH 1-3, acetate for pH 4-5, phosphate for pH 6-7, Tris-HCl for pH 8-9 and Glycine-NaOH for pH 10-12. The data was fitted into non-linear regression analysis of LB plot.

For studying stability of Amy Strplo 716 at different pH, the enzyme was incubated in respective buffers (4.0 to 11.0) for 30 minutes at 25°C, suitable aliquot was removed and residual activity was determined.

pK_a of amino acids at the active site

To determine the probable residues at the active site of the enzyme, Km and V_{max} of *Strep-α-amyl* enzyme were estimated at different pH ranging from 4.0 to 11.0. 2µg protein was used for each assay. Ln (V_{max}/ K_m) Vs pH was plotted to determine pKa value.

Enzymatic hydrolysis of soluble starch with Amy Strplo 716

To determine the mode of action of the enzyme, the degree of polymerization (DP) of the products was determined by HPLC (Nirmala *et al*, 2001). The hydrolysis reaction contained 2 IU of Amy Strplo 716, 2% soluble starch in 50 mM phosphate buffer of pH 8.0. The reaction mixture was incubated at 45°C at various time intervals of 1 h to 24 h. The progress of reaction was monitored at different time intervals. After specific time interval, the reaction mixture was neutralized by the addition of 1 N HCl followed by boiling the reaction for 5 min. 3 volumes of absolute ethanol was added to the hydrolysate and the mixture was kept at 4°C for 6 h for precipitation. After

centrifugation at 10,000 rpm, the supernatant was concentrated in a rotavap and the product was dissolved in ultra pure water and filtered through a 0.22 µM membrane. 20 µL sample was loaded on µBondapak NH2 (3.9 x 300 mm, 10 µm) Shimadzu HPLC column equipped with a RI detector. The amounts of oligosaccharides (DP1-DP7) were quantified by peak integration, with standards detected using a refractive index detector. The HPLC conditions for the sample analysis are as follows: Solvent system: Acetonitrile: water (70:30); Temperature: 25°C; Flow rate: 1 ml/ min, isocratic; Injected volume:20 µL. Standards used: Maltose to heptaose.

Optimum temperature and temperature stability of Amy Strplo 716

The assay of Amy Strplo 716 enzyme was performed at different temperature ranging from from 37°C to 47°C in 50mM glycine-NaOH pH 9.0. 2 µg protein was used for each assay.

Determination of temperature stability of purified Amy Strplo 716 was studied by incubating the enzyme at pH 9.0 for 5 minutes at various temperatures ranging from 45°C to 60°C in 50 mM glycine-NaOH pH 9.0 and suitable aliquot was removed and residual activity was determined.

Activation Energy

Km and Vmax were determined for Amy Strplo 716 at pH 9.0 at various temperatures (37°C to 45°C). The energy of activation; Ea, of the enzyme was calculated from the slope of the plot of ln V_{max} *V*s 1000/*T*. *E*a =−slope × *R* (*R*, gas constant = 8.314×10^{-3} kJ/mol).

Effect of metal ions on Amy Strplo 716

The enzyme assay was carried out in presence of metal ions such as Al, Cu, Co, Mg, Mn, Ca, Zn and Hg at 1mM concentrations of metal ions. Reversibility of the inhibition of enzyme activity due to EDTA (2mM) was checked by adding 1mM Calcium ions.

Effect of denaturants (Guanidine Hydrochloride)

The enzyme assay was carried in presence of various concentrations (0.25, 0.5, 0.75, 1, 1.5, 2 and 3.0 M) of guanidine hydrochloride.

Chemical Modification of Amy Strplo 716

Reaction with p-hydroxymercurybenzoate (pHMB)

The enzyme solution (60 µg) in 10 mM Tris HCl buffer solution, pH 7.6, was incubated with varying concentrations of pHMB (1, 5 and 10 mM) at 25°C. Aliquots were removed at different time intervals and the residual activity was determined under standard assay conditions.

Reaction with 2, 2'-dithiobisnitrobenzoic acid (DTNB)

The enzyme solution (1.65 µM, 1ml) in 50 mM potassium phosphate buffer, pH 7.8, was incubated with 1.0 mM of DTNB (effective concentration) at 30°C for 45 min. The modification reaction was followed by monitoring the absorbance at 412nm and the number of sulfhydral groups modified was calculated by using a molar absorption co-efficient of 13,600 $M^{-1}cm^{-1}$.

Determination of disulfide bond with DTNB after reduction

To a test tube containing 1.44 mg of urea, (0.1 ml) 0.1M sodium EDTA, 0.5-1.0 ml of protein sample (200 µg), 1ml of 2.5% $NaBH_4$, water to make volume 3 ml, and a drop of octyl alcohol as an antifoaming agent were added. The samples were incubated at 37°C and reduction was allowed to proceed for 30min. After this, 0.5 ml of 1M KH_2PO_4 containing 0.2 M HCl was added and nitrogen was bubbled through the solution for 5 min. Finally, 0.5 ml of 0.01M DTNB was added and the volume was made to 6 ml with water. Nitrogen was bubbled for 2 minutes and tube was stoppered, ensuring that the gas space was filled with nitrogen. The preparation was stabilized for 15 min, the absorbance was determined at 412 nm. Blanks containing all reactants except the protein solution were subtracted. A molar absorptivity of 12,000 $M^{-1}cm^{-1}$ was used for calculating the number of sulfhydral groups formed after reduction.

Modification of Arginine Residues with p-nitrylphenylglyoxal (PNPG)

The enzyme (60µg) in 10 mM Tris HCl buffer, pH 7.6, was incubated with varying concentrations of pNPG (1mM-10mM) at 30°C (Takashi, 1968). Aliquots were removed at suitable intervals and the residual activity was determined under standard assay conditions.

Modification of Tryptophan Residues with N-bromosuccinimide (NBS)

The reaction was carried out by titrating 1ml of enzyme solution (200 µg/ml) with freshly prepared NBS (2 mM) in the same buffer (Spande and Witkop, 1967). The NBS mediated reaction was followed by monitoring the decrease in the absorbance at 280 nm. The reagent was added in installments (5 µl each) till the protein: NBS ratio reached 1:10. Similar additions were carried out in a tube containing duplicate sample and after each addition, an aliquot was removed and checked for residual activity under standard assay conditions. The number of tryptophans modified was determined spectrophotometrically, by assuming a molar absorption co-efficient of 5500 $M^{-1}cm^{-1}$ for the modified Trp at 280 nm. The absorption spectral measurements were made using a Shimadzu UVB101 PC double-beam spectrophotometer.

Modification of histidine using diethylpyrocarbonate (DEPC)

Reaction mixture of 1ml contained 60 µg of Amy Strplo 716 in 10 mM Tris-HCl buffer, pH 7.6 with various concentrations of DEPC freshly prepared in absolute ethanol (7, 14, 21mM) and was incubated for time ranging from 0 to 15 minutes at an interval of 5 minutes. The ethanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. Amy Strplo 716 samples incubated in the absence of DEPC served as control. The reactivation of the inactivated Amy Strplo 716 enzyme was carried out by incubating the enzyme, DEPC treated enzyme with hydroxylamine hydrochloride (25 mM) for 10 minutes.

Modification of Tyrosine Residues with N-acetyl imidazole (NAI)

This was performed as described by Riordan *et al* (1965). The enzyme (60 µg) was taken in 10 mM Tris HCl buffer pH 7.6 was incubated in presence of 20 mM NAI. The enzyme incubated in the absence of NAI served as control. Aliquots were withdrawn and assayed for the residual activity of α-enzyme.

Modification of Lysine Residues

Citraconylation

The purified Amy Strplo 716 (60 µg) was taken in 10 mM phosphate buffer, pH 7.6, was treated with 1mM, 5 mM, 10 mM and 15mM Citraconic anhydride at 25 °C.

The amino groups of Amy Strplo 716 were reversibly modified by citraconic anhydride according to Dixon and Perham (1968). After each addition, an aliquot was removed and assayed for residual Amy Strplo 716 enzyme activity.

Substrate protection studies

Substrate protection studies were carried out by adding 250 µl of 10 mg/ml of starch to the enzyme before treatment with different chemical modifiers and then assaying the modified enzyme with proper controls.

Circular Dichroism (CD) measurement

The CD spectra of the enzyme were recorded on a J-175 Spectropolarimeter with a PTC343 Peltier unit (Jasco, Tokyo, Japan) at 25°C in a quartz cuvette. Each CD spectrum was accumulated from three scans at 50 nm/min with a 1 nm slit width and a time constant of 1 s for a nominal resolution of 0.5 nm. Far UV CD spectra of the enzyme were collected in the range of wavelengths of 200-250 nm using a cell path length 0.1 cm for monitoring the secondary structure. All spectra were corrected for buffer contributions and observed values were converted to mean residue ellipticity. The tertiary structure of the enzyme (1mg/ml) was monitored with near UV CD spectra in the wavelength 250-300 nm using path length 1 cm. Results were expressed as mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ defined as

$$MRE = M\,\theta_\lambda / 10\,d\,c\,r$$

Where M is the molecular mass of the protein, θ_λ is CD in millidegree, d is the path length in cm, c is the protein concentration in mg/ml and r is the average number of amino acid residues in the protein. Secondary structure elements were calculated by using CD pro software (Sreerama *et al*, 1999).

Steady State Fluorescence Measurement

Intrinsic fluorescence of the enzyme was measured using a Perkin-Elmer Luminescence spectrometer LS50B connected to a Julabo F20 water bath. The protein solution of 100µg/ml was excited at 295 nm and the emission was recorded in the range of wavelength 300-400 nm at 30 °C. At 295 nm, major fluorescence is due to the

tryptophan residues. The overall conformation can be monitored under different conditions. The slit widths for the excitation and emission were set at 7.0 nm, and the spectra were recorded at 100 nm/min. To eliminate the background emission, the signal produced by either buffer solution or buffer containing the appropriate quantity of denaturants was subtracted.

Decomposition of fluorescence spectra

The decomposition of trp fluorescence spectra was carried out using PFAST program (http://pfast.phys.uri.edu/pfast/) developed based on the SIMS and PHREQ algorithm. (Burstein *et al*, 2001; Wang *et al*, 2008).

Solute quenching studies

Fluorescence measurements were performed for native and denatured protein with different quenchers like acrylamide (5 M), iodide (5 M) and cesium ions (5 M) (charged quenchers), on Perkin-Elmer LS 50B spectrofluorimeter at 30°C. Amy Strplo 716 samples of < 0.1 OD were excited at 295 nm and emission spectra were recorded in the range of 300–400 nm. These experiments were carried out using the protein solution of concentration 100 μg/ml. Slit widths of 7.0 nm each were set for excitation and emission monochromators. Small aliquots of the quencher stocks were added to protein samples and fluorescence spectra were recorded after each addition. Iodide stock solution contained 0.2 M sodium thiosulphate was added to prevent formation of tri-iodide (I^{-3}). For quenching studies with denatured protein, the protein (100 μg/ml) was incubated with 6 M Gdn-HCl overnight at room temperature. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

The steady-state fluorescence quenching data obtained with different quenchers were analyzed by Stern–Volmer (Eq. 1) and modified Stern–Volmer (Eq. 2) equations in order to obtain quantitative quenching parameters (Lehrer, 1971)

$$F_0/Fc = 1 + Ksv\,[Q].....(1)$$

$$F_0/\Delta F = f^{-1}a + 1/\,[K_a f_a(Q)].....(2)$$

Where F_0 and Fc are the relative fluorescence intensities in the absence and presence of the quencher, respectively, (Q) is the quencher concentration, K_{sv} is Stern–Volmer

quenching constant, $\Delta F=F_0-Fc$ is the change in fluorescence intensity at any point in the quenching titration, K_a is the quenching constant and f_a is the fraction of the total fluorophores accessible to the quencher. Equation (2) shows that the slope of a plot of $Fo/\Delta F$ versus $(Q)^{-1}$ (modified Stern–Volmer plot) gives the value of $(K_a f_a)^{-1}$ and its Y-intercept gives the value of f_a^{-1}.

For quenching studies with denatured protein, the protein was incubated with 6 M GdnHCl overnight at room temperature. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

Lifetime measurement of fluorescence decay

Lifetime measurements were carried out on Edinburgh Instruments' FLS-920 single photon counting spectrofluorimeter. An H_2 flash lamp of pulse width 1.0 ns was used as excitation source and a Synchronization photomultiplier was used to detect the fluorescence. The diluted Ludox solution was used for measuring Instrument response function (IRF). The samples (1mg/ml) were excited at 295 nm and emission was recorded at 357 nm. Slit widths of 15 nm each were used on the excitation and emission monochromators. The resultant decay curves were analyzed by a reconvolution fitting program supplied by Edinburgh Instruments. The bimolecular quenching constant K_q was calculated as $K_q = K_{sv}/\tau$ where τ is the average life time of the protein in the absence of a quencher. The average life time was calculated using the formula,

$$(\tau) = \Sigma i\, \alpha i \tau^2 i \,/ \Sigma i\, \alpha i \tau i, \text{ where } i = 1,2\ldots\ldots \quad (3)$$

ANS-binding Assay

The formation of intermediate state of Amy Strplo 716 under denaturing condition at variable pH 1.0, 3.0, 5.0, 7.0, 9.0 and 11.0 was analyzed by the hydrophobic dye (1-anilino-8-naphthalenesulfonate) binding. The dye was prepared in methanol and the vial was covered to protect the solution from light. The final ANS concentration used was 50 μM, excitation wavelength, 375 nm and total fluorescence emission was monitored between 400-550 nm. Reference spectrum of ANS in each buffer of respective pH was subtracted from the spectrum of the sample.

Results and discussions

Production and Purification of Amy Strplo 716 from *Streptomyces lonarensis* strain NCL 716

The *Streptomyces lonarensis* strain NCL 716 is an alkalophilic strain and the maximum enzyme production was obtained in the pH range of 9.0-10.0. The α-amylase was produced as an extracellular enzyme in a medium with starch as a prime influential factor and as an inducer of the enzyme. The yield of the enzyme was 29.7 IU/ml, which is comparable with the other alkalophilic strains (table 4.1).

Table 4.1: Alkaline amylase yield from various bacterial sources

Organism	Enzyme produced (IU/ml)	Reference
Streptomyces sp.KSM-9	0.85	Nakai *et al*, 1986
Bacillus species GM8901	0.29	Kim *et al*, 1995
Bacillus strain HUTBS71	72	Farouk-Al-Quadan, 2009
Bacillus sp. PN5	57.32	Saxena. *et al*, 2007
Bacillus megaterium	7.05	Khan *et al*, 2011
Bacillus subtilis strain WB600	44.1	Yang *et al*, 2011
Streptomyces lonarensis strain NCL 716	29.7	NCL, Pune, India.

For purification, the ammonium sulphate precipitated (90% saturation), dialysed, and concentrated supernatant of the culture broth was subjected to the preparative PAGE. The enzyme activity located in the fraction of poly-acrylamide gel after elution showed a single band on native PAGE (Fig 4.1). The spectroscopic impurities in the protein were removed by subjecting the protein to DEAE –cellulose chromatography. The final recovery, fold purification and specific activity of the pure enzyme was 46%, 3.12 and 7400 U/mg (Fig. 1.1), respectively.

Table 4.2: Purification table of Amy Strplo 716

Sr. No	Purification table	Activity (IU)	Protein (mg)	Specific Activity (U/mg)	Fold Purification	Recovery
1	Crude	2970	58	5120	1	100
2	$(NH_4)_2SO_4$ precipitation	2230	43	5184	1.34	75
3	Preparative PAGE (10 batches)	1370	18	7400	3.12	46

Molecular weight determination

Native and SDS-PAGE of the purified enzyme showed a single band on both the gels (Fig 4.1). The molecular mass was estimated as 37,000 Da on SDS-PAGE.

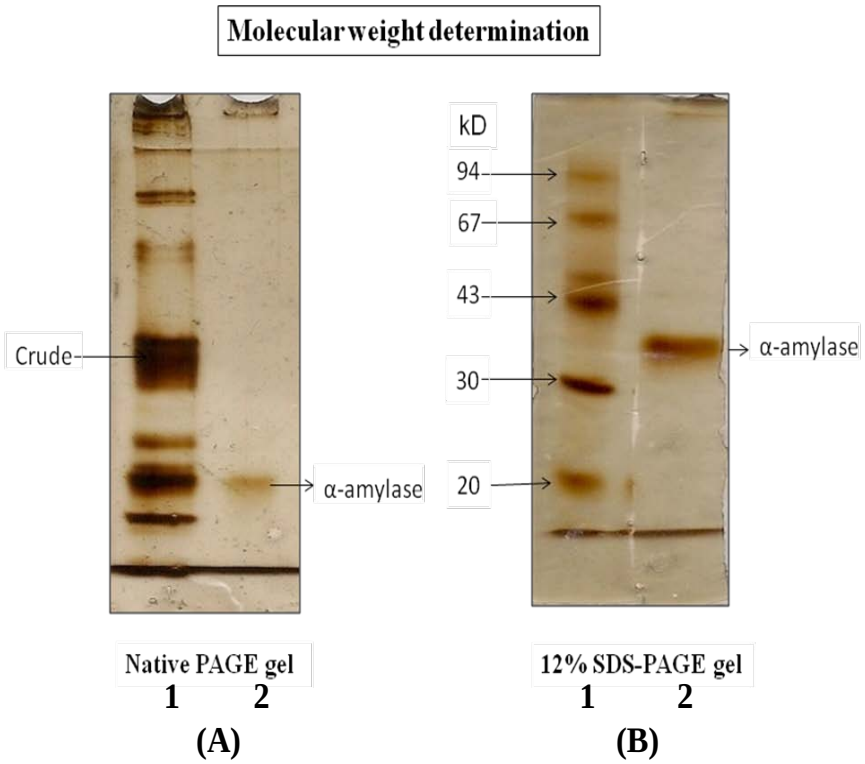

Figure 4.1: **Native PAGE gel**: Crude Amy Strplo 716 (A) Pure Amy Strplo 716 (10 µg) in (B) **SDS PAGE 12 % (w/v)** SDS-PAGE High molecular weight marker (kDa) lane (C); Pure Amy Strplo 716 (20 µg) (D). Protein bands were visualized with silver staining. (Fig.4.1).

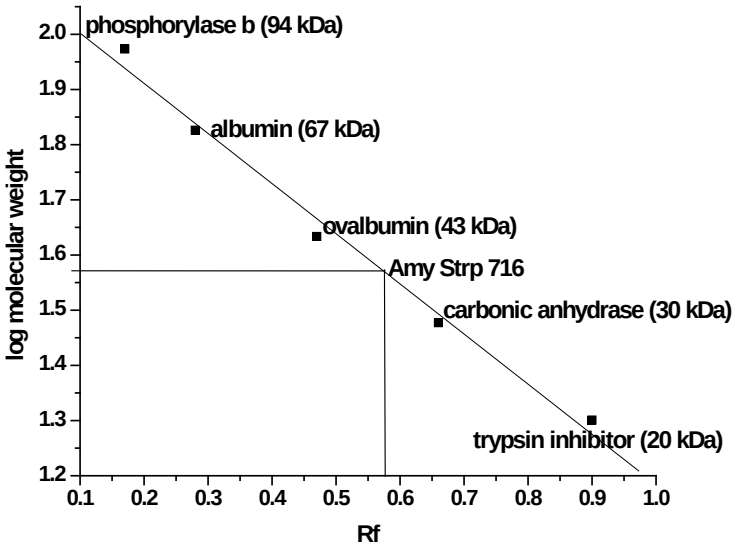

Figure 4.2: Molecular weight determination plot of Rf (determined from SDS-PAGE) against log molecular weight

Catalytic properties

K_m and V_{max} are important coefficients in enzyme kinetics. Smaller Km values indicate more firm enzyme binding to its substrate. K_m is independent of enzyme concentration and is a true characteristic of the enzyme under defined conditions of temperature, pH etc (Negi and Banerjee, 2009). In the present research work, the V_{max} and K_m of Amy Strplo 716 were derived from the Lineweaver Burke plot using substrates starch, amylose and amylopectin.

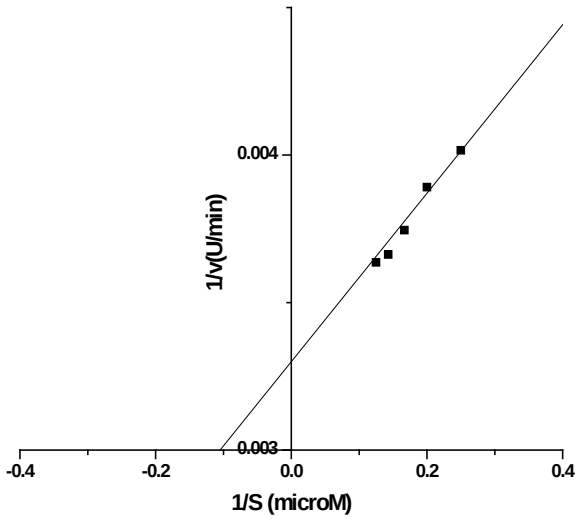

Figure 4.3: Lineweaver - Burk plot showing K_m and V_{max} of Amy Strplo 716 with soluble starch.

Table 4.3 shows the values of K_m and V_{max} for Amy Strplo 716 with three substrates. The enzyme showed similar values of K_m with starch and amylopectin and slightly lower K_m value with amylose indicating little higher affinity for amylase. This could be due to the linear arrangement of glucose molecules with α, 1-4 linkage in amylose. The V_{max} for all three substrates are same.

α-amylase from *B.licheniformis* showed V_{max} and K_m values of 2778 U/mg/min and 8.3mg/ml, respectively. Gangadharan *et al*, (2009) reported K_m and V_{max} values for soluble starch to be 4.11 mg/min and 3.076 mg for α-amylase from *B. amyloliquefaciens*, respectively. For α-amylase from *Lactobacillus manihotivorans*, K_m and V_{max} values were 3.44 mg/ml and 0.45 mg hydrolyzed starch/ml/min, respectively (Goyal *et al*, 2005).

Table4.3: K_m and V_{max} values for Amy Strplo 716

Substrate	**K_m(mg/ml)**	**V_{max} (U)**
Starch	2.0	2.43
Amylose	1.422	2.42
Amylopectin	2.0	2.268

Optimum pH and pH stability of Amy Strplo 716

Effect of pH

The enzyme exhibited optimum activity at pH 9 and dropped down gradually below pH 9.0 till pH 6.0. The enzyme showed drastic inactivation at pH 10.0 and above.

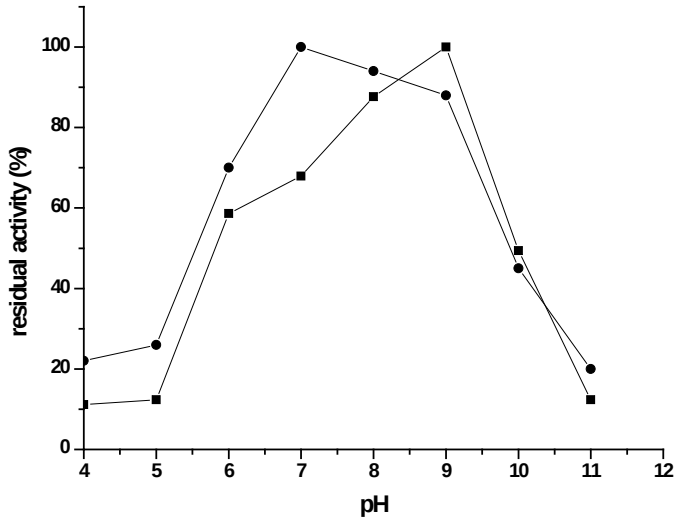

Figure 4.4: Effect of pH on activity and stability on Amy Strplo 716 (■), the purified protein was assayed for Amy Strplo 716 activity at 45°C in 50 mM buffer of respective pH. 100% of relative activity corresponds to 16.2 IU/ml of enzyme. **(●), t**he purified protein was incubated in respective buffers for 30 minutes at 25°C, suitable aliquot was removed and residual activity was determined.

pH stability

To determine the pH stability of Amy Strplo 716, the enzyme was incubated in buffers of various pH for 30 min and the residual enzyme activity was assayed at 45°C for 30 min. As shown in the figure, the enzyme is fairly stable in the pH range 7.0 to 9.0 and loses activity beyond this range. At pH 6.0, 70% residual activity was observed. At pH 4.0, only 22% of residual activity was obtained. The enzyme has more stability in the alkaline pH range.

Most of the amylases reported so far exhibit pH optima in acidic or neutral ranges. Few reports are available on alkaline amylases from *Streptomyces* sp. Nakai *et al* have reported an alkaline amylase from *Streptomyces sp KSM-9*. Moriyama *et al* and Syed *et al* have reported alkaline amylases from *Streptomyces sp*. The present *Streptomyces* strain is the fourth report of alkaline amylase from *Streptomyces.*

Effect of temperature

The Amy Strplo 716 activity at pH 9.0 was measured at various temperatures in the range of 30°C-60°C. The enzyme showed maximum activity at

45°C. The residual activity of Amy Strplo 716 was 89% at 47°C and drastically declined to 51% at 55°C.

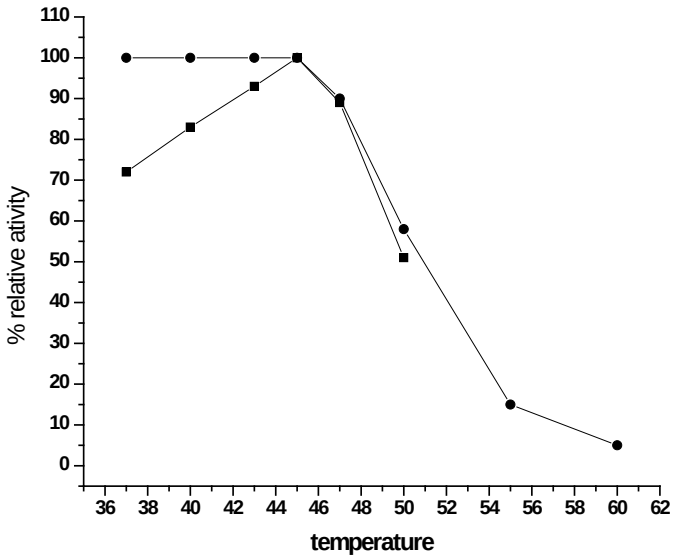

Figure 4.5: Effect of temperature on activity and stability on Amy Strplo 716 (■)**,** the purified protein was assayed for Amy Strplo 716 activity at temperatures from 37°C to 47°C in 50 mM glycine-NaOH pH 9.0. Relative activity of 100% corresponds to 16.2 IU of enzyme. (●)**,** the purified protein was incubated at pH 9.0 for 5 min at various temperatures from 45°C to 60°C, suitable aliquot was removed and residual activity was determined.

The stability of Amy Strplo 716 was checked at pH 9.0 at various temperatures. The enzyme was stable at 45°C for more than 2 h but rapidly lost activity above 45°C. The relative activity decreased to 58% at 50°C. There was a drastic decrease to 15% at 55°C and to 5% at 60°C.

Analysis of end products of enzymatic hydrolysis of soluble starch with Amy Strplo 716

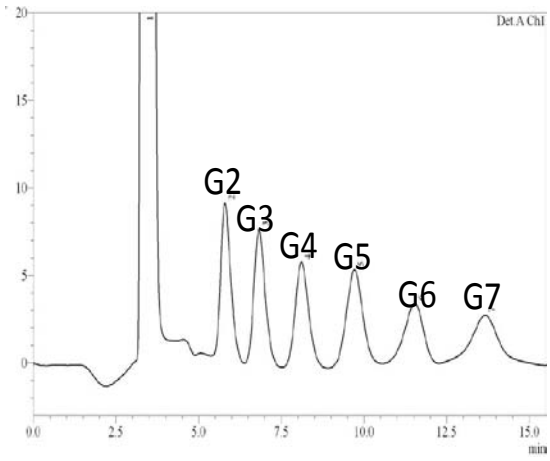

Fig 4.6(a) standards

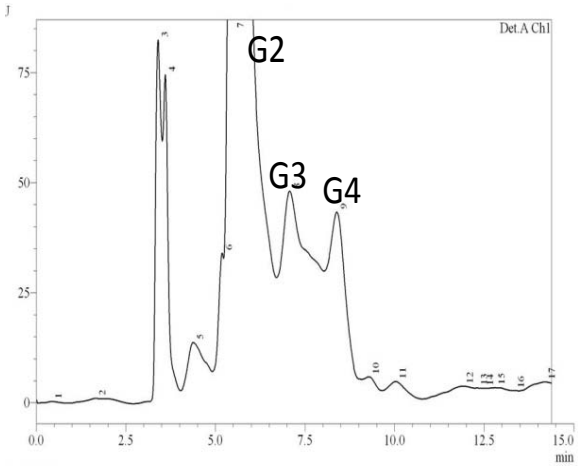

Fig 4.6(b)1h hydrolysis

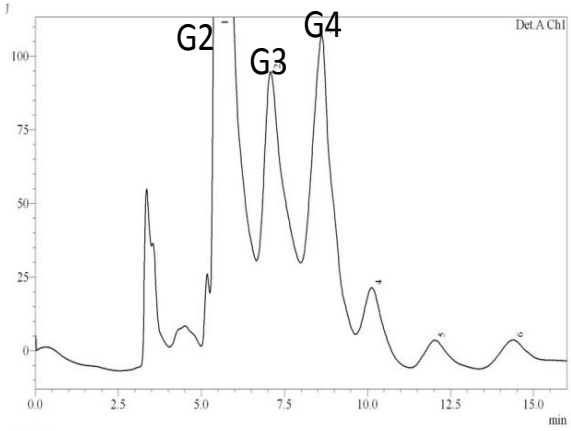

Fig 4.6(c)12h hydrolysis

Figure 4.6(a) HPLC standards used: G2, G3, G4, G5, G6, G7 and their retention times are **5.791, 6.817, 8.105, 9.709, 11.532** and **13.665**, respectively. **(b)** Formation of end products G2, G3 and G4 on starch hydrolysis after 1 h and **(c)** after 12 h.

The HPLC profile of the starch hydrolysate for the Amy Strplo 716 showed the presence of maltose, maltotriose and maltotetraose as the end-product. The amount of G2, G3 and G4 increased to maximum level within 12 h. The first report of α-amylases producing specific sugars was from the culture filtrates of *Pseudomonas stutzerii* (Robyt and Ackerman, 1971). Subsequently, there have been several reports of α-amylases

producing specific sugars ranging from G2 to G6, mainly from the genus *Bacillus* and *Pseudomonas*, most of which are active at neutral or alkaline pH. Till date there are only two reports of maltotriose being produced by neutrophilic *Streptomyces* strains (Syed *et al*., 1999; Wako *et al*., 1979) and one report each of maltotetraose. Thus Amy Strplo 716 can be considered to have a potential in starch hydrolysis to form maltose, maltotriose and maltotetraose.

Determination of pKa of amino acid residues at the active site

The K_m and V_{max} of the enzyme were determined at different pH from 5.0 -11.0 and the pH-activity profile was obtained by plotting log (V_{max}/K_m) against pH for getting an idea about the amino acid residues involved at the active site (Fig 4.3.). The profile revealed the participation of ionizable groups with pKa values of 7.7 and 8.7 indicating probable involvement of histidine and/or cysteine and arginine respectively, at the active site (pKa values can change slightly due to change in the microenvironment). Chemical modification of the amino acids with the group specific reagents was taken up for detailed studies of the active site, which is described later in this chapter.

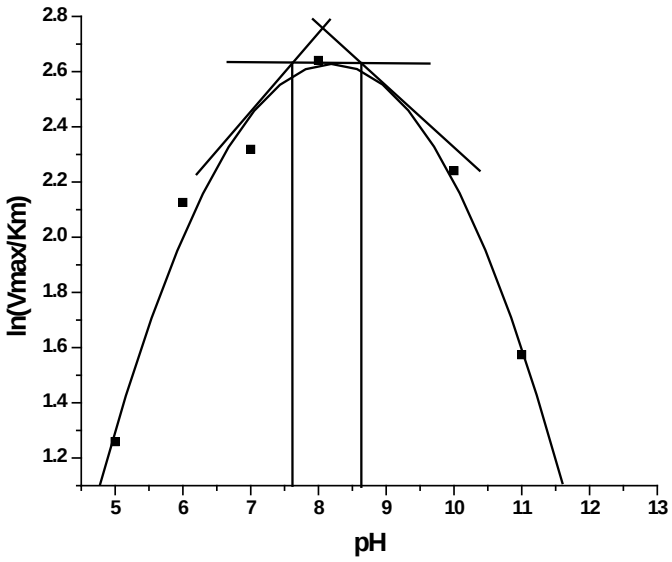

Figure 4.7: pH activity profile for Amy Strplo 716: The K_m and V_{max} values of the enzyme (15 µg) were determined at different pH (5-11) by using 1mg to 10 mg of starch substrate and fitting the data to linear regression using Line weaver-Burk plots. Buffers used for assays at different pH have been mentioned in Materials and Methods.

Activation Energy

The energy of activation *E*a, of the enzyme with soluble starch substrate was calculated from the slope of the plot of ln V_{max} *V*s 1000/*T*, as *E*a =−slope × *R* (*R*, gas constant = 8.314×10−3 kJ/mol) which was estimated to be 18.36 KJ mol^{-1}.

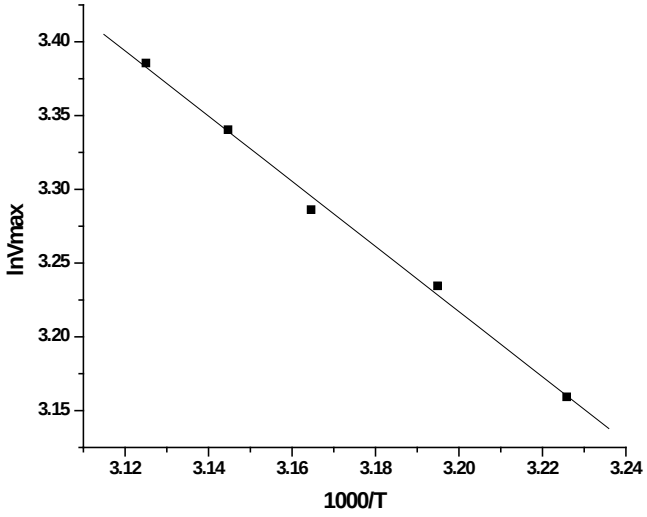

Figure 4.8: Energy of activation of Amy Strplo 716 with starch substrate. Plot of ln V_{max} *V*s 1000/*T*, *E*a =−slope × *R* (*R*, gas constant = 8.314×10^{-3} kJ/mol). 2 µg of enzyme was taken for the assay of Amy Strplo 716 enzyme. The reaction mixtures containing different concentrations of substrates were incubated at different temperatures. Values of V_{max} were taken from Lineweaver-Burk plots. The activation energy was estimated to be 18.36 KJ mol^{-1} for alkaline Amy Strplo 716.

Table 4.4:Comparative account of reported Activation energy of some alpha amylases from different sources.

Substrate	Activation Energy (E_a)	Amy Strplo 716 Source
Maltoheptaose	33.3 KJ mol^{-1}	Serum
Blue Starch	54.4 KJ mol^{-1}	Serum
Soluble Starch	44 $KJmol^{-1}$	*Thermomyces lanuginosus*
Soluble Starch	25.14 $KJmol^{-1}$	*Bacillus licheniformis*
Soluble Starch	51 $KJmol^{-1}$	*Bacillus licheniformis CUMC305*
Soluble Starch	18.36 KJ mol^{-1}	*Streptomyces lonarensis* strain NCL 716

From the table 4.4 it can be proposed that, Amy Strplo 716 with a lower activation energy value can find a potential and significant application in industries.

Effect of metal ions on the Amy Strplo 716 activity

The Amy Strplo 716 activity was assayed in presence of 1mM metal ions. Table 4.5 shows effect of metal ions on the enzyme activity. Drastic inactivation of the amylase activity was observed in presence of Hg^{2+} (17% residual activity) as also reported for other amylases. Increase in the residual activity up to 129 % in presence of Mg^{2+}, 125% in presence of Co^{2+} and 115% in presence of Ca^{2+} was observed. This could be due to the stabilization of active site geometry. The enzyme lost 50 % of activity in presence of 2 mM EDTA. Almost 85% activity was regained on addition of 1mM $CaCl_2$ to the EDTA treated enzyme, indicating that Ca^{2+} was required for enzyme activity. It can also be proposed that the enzyme already has Ca^{2+} bound to it.

Most amylases require calcium for stability and are readily inactivated by EDTA. *Streptomyces D1* amylase activity was stimulated in presence of calcium ion (5 and 10 mM). The α-amylase from *Bacillus* sp. KSM-1378 (Cordeiro *et al*, 2002) and *Bacillus firmus* (Zhang *et al*, 2007) were strongly inhibited by Ni^{2+}, Cd^{2+}, Zn^{2+} and Hg^{2+}. The α-amylase from *Thermus* sp. was strongly inhibited by Cu^{2+} and Fe^{2+} (Shen *et al*, 1998) and the α-amylase from *B. subtilis*, *B. Amyloliquefaciens* I, and *B amyloliquefaciens* II were strongly inhibited by Zn^{2+}, Ag^{+}, Cu^{2+} and Fe^{2+} (Elif *et al*, 2000). The amylase enzyme from *Bacillus Strain, GM8901* required metal ions such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Ag^{+}, Zn^{2+}, and Fe^{2+} for its enzyme activity and was inhibited by 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (Chakraborty *et al*, 2009).

Table 4.5: Percent residual activity of Amy Strplo 716 by different metal ions at 1mM concentration.

Metal ions (1mM)/EDTA	**Residual activity (%) Amy Strplo 716**
Al^{3+}	50
Mg^{2+}	129
Co^{2+}	125
Ca^{2+}	115

Hg^{2+}	17
Cu^{2+}	55
Zn^{2+}	69
2mM EDTA	50
2mM EDTA + 1 mM $CaCl_2$	85

Effect of denaturant (GndHCl) on Amy Strplo 716 Almost 80% inactivation of the enzyme was observed even in presence of 0.25 M Gdn-HCl. (Fig 4.9). The observed loss in the activity of Amy Strplo 716 could be due the changes in the active site of the protein.

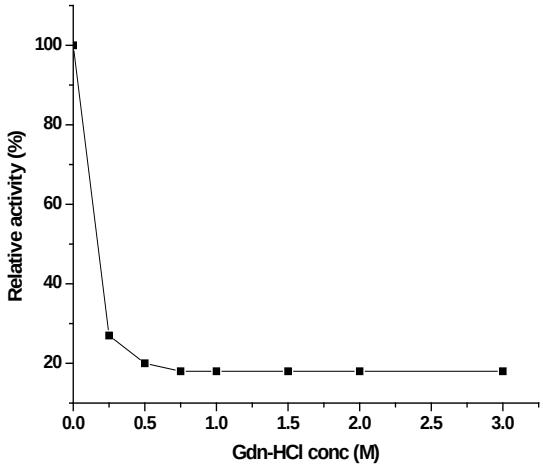

Figure 4.9: Effect of denaturant (GdnHCl) on the Amy Strplo 716 activity. (■), the purified protein was treated with increasing amount of Gdn-HCl (from 0.25-3.0 M) and then assayed for Amy Strplo 716 activity at 45°C at pH 9.0. 100% of relative activity corresponds to 16.2 IU/ml of enzyme.

The interaction of Gdn-HCl with carboxylate groups of the protein has been experimentally proved on the basis of (1) inhibition by low concentrations of GdnHCl of enzyme which essentially require carboxyl groups for the catalytic activity, (2) failure of the carboxyl specific Woodward's reagent K to react specifically with the carboxyl groups to the proteins/enzymatic pretreated with GdnHCl. (Ghatge *et al*., 1993) The spectral analysis of the reaction between WRK and Gdn-HCl exhibited that

Gdn-HCl preoccupies the sites which are accessible to WRK. This also could be due to the positively charged arginine-like guanidine moiety, electrostatically reacts with the carboxyl groups of the protein. (Woodward *et al*.,1990). Gdn-HCl can be successfully utilized for determining the functional role of the carboxylate residues in the protein with an added advantage of being inexpensive.

Hence it can be suggested that the catalytic site of Amy Strplo 716 has carboxylate groups.

Chemical Modification of Amy Strplo 716

Chemical modifications of the reactive amino acid side chains in protein have found immense applications in modern protein science such as analysis of amino acid, sequencing, peptide mapping and mass spectroscopy. One of the vital and conventional applications includes the use of chemical reagents for structure and function applications, mainly in identifying the residues in enzyme active sites. Chemical modification studies can be used as an initial step to determine the probable involvement of residues in a specific biological function, and when coupled with mutagenesis studies yield more confirmatory and extended initial observations.

To know the active site residues, chemical modification of the enzyme was carried out with the amino acid specific reagents. Out of the seven chemical modifiers used in this work, NBS (for tryptophan), DEPC (for histidine), pHMB (for cysteine), phenyl glyoxal (for arginine) and Citraconic anhydride (for lysine) showed inhibition of the enzyme activity (Table 4.6).

Chemical modifiers like N-acetylimidazole (20mM) and Woodwark's reagent (WRK) (20 mM) had no major effect on the activity of Amy Strplo 716 activity. Almost 80 - 85% of residual activity was detected after modification of the enzyme. In case of WRK, the modification reaction has to be carried out at pH 6.0, while the optimum pH of Amy Strplo 716 is pH 9.0. Only 15% inactivation of the enzyme was observed in presence of 20 mM WRK.

Table 4.7: Summary of the chemical modification studies of Amy Strplo 716

Chemical modifier	Residues modified	Residual activity (%)
NBS (2 mM)	Tryptophan	43
NAI (20 mM)	Tyrosine	80
Citraconic anhydride (15 mM)	Lysine	50
p-hydroxy mercuricbenzoate (1mM)	Cysteine	49
WRK (20mM)	Carboxylate	85
Phenyl glyoxol (1mM)	Arginine	52
DEPC (21mM)	Histidine	45

Reaction with 2, 2' –dithiobisnitrobenzoic acid (DTNB)

Based on the modification of the enzyme with DTNB, the number of free Cys residues in Amy Strplo 716 was estimated to be one. Modification of the enzyme after reduction of the denatured protein yielded seven cysteine residues. Hence, there could be 3 disulfide bonds in the enzyme.

Reaction with p-hydroxymercurybenzoate (pHMB)

Incubation of Amy Strplo 716 with varying concentrations of *p*-HMB as 1 mM, 5 mM and 10 mM led to decrease in the residual activity to 49%, 35% and 21% respectively. The enzyme incubated with soluble starch substrate (2.5 mg) prior to chemical modification with 1 mM pHMB showed 69% of the residual activity. This suggests the presence of cysteine residue at the active site of Amy Strplo 716.Substrate protection of 69% was obtained when enzyme was incubated with 2.5 mg of starch prior to the modification with *p*HMB.

Modification of Tryptophan Residues with N-bromosuccinimide (NBS)

Modification of Trp with NBS was accompanied by a decrease in the absorbance of the protein at 280 nm. Based on the molar absorption co- efficient of

5500 M^{-1} cm^{-1} and molecular mass of enzyme as 37,000 Da, the number of tryptophan residues in the native and denatured protein was estimated to be three and eight, respectively. This indicates that three tryptophans are present on the surface of the protein while five tryptophan residues are present in the interior of it.

Oxidation of the Amy Strplo 716 by N-bromosuccinimide resulted in substantial loss of its activity, suggesting a role of tryptophan residues in the catalysis of the enzyme. Estimation of the activity of the NBS modified enzyme indicated one tryptophan residue to be essential for the enzyme activity (Fig. 4.5). Substrate protection of 99% was seen when enzyme was incubated with 2.5 mg of starch prior to modification with (2 mM) NBS.

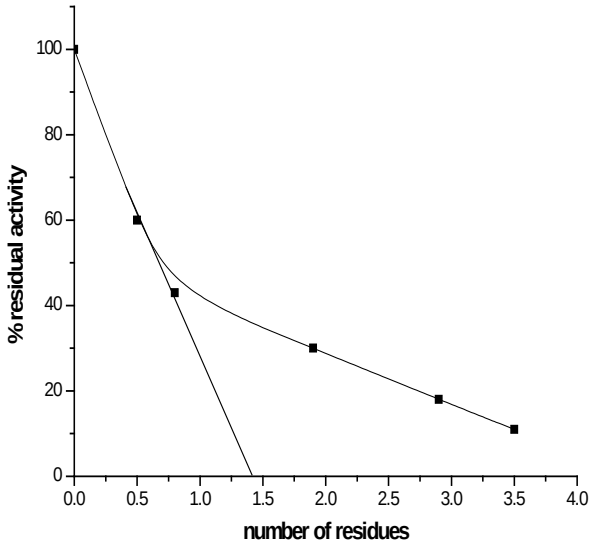

Figure 4.10: Plot of percentage residual activity against number of Trp residues modified as determined by spectroscopic studies. The reaction mixture contained 250 µg/ml of Amy Strplo 716 in pH 7.0 in 10 mM Tris-HCl buffer and NBS. In the duplicate reaction mixture, after each addition of NBS, suitable aliquot was taken at regular intervals and residual activity was determined.

Modification of histidine using diethylpyrocarbonate (DEPC)

Modification of the enzyme with varying concentrations of DEPC as 7 mM, 14 mM and 21 mM showed drop in the activity initially within five minutes. Later, the rate of inactivation had slowed down. About 45 % inactivation of the enzyme was observed in presence of 21 mM DEPC.

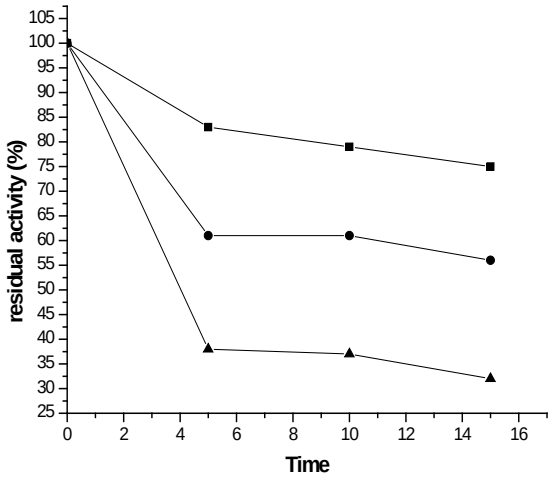

Figure 4.11: Chemical modification of Amy Strplo 716 with DEPC. The reaction mixture contained 60 µg of the purified Amy Strplo 716 with varying concentrations as **7 mM (■), 14 mM (●) and 21 mM (▲) DEPC.** Samples were withdrawn at an interval of 5 minutes and the residual activity was determined.

The inactivation of DEPC modified enzyme (45%) was reversed to 102% with hydroxylamine. This confirmed the specific modification of histidine. Substrate protection of 87% was obtained when enzyme was incubated with 2.5 mg of starch prior modification with 21 mM DEPC. Above results suggest that Tryptophan, Cysteine, Histidine are important for the binding of substrate and catalysis activity of the amylase activity.

Substrate protection against modification of the enzyme with p-nitrophenylglyoxal (PNPG), N-acetyl imidazole (NAI) and citranonic anhydride was not observed indicating that the inactivation could be due to the structural change in the protein.

Table 4.8: Substrate protection studies during chemical modification with reagents NBS, DEPC and pHMB

Amino acid modified	Substrate Protection Studies	
	Conditions	**residual activity (%)**
None	E+Substrate	100
Tryptophan	E +NBS (2mM)	43
	E+S(2.5mg)+NBS(2mM)	99
Histidine	E+ DEPC (21mM)	45
	E+S(2.5mg)+DEP(21mM)	87
	E+DEPC(21mM)+hydroxylamine hydrochloride (25 mM)	100
Cysteine	E+pHMB(1mM)	49
	E+S(2.5mg)+pHMB(1mM)	69

Circular dichroism (CD) measurements

Far UV CD-spectrum of Amy Strplo 716 at pH 7.6 showed a trough with minima at 222 nm indicating predominance of β-sheet structures in the protein (Fig. 4.12). The CD spectra exhibited characteristic peaks (intensive positive peak at around 190 nm and two negative double humped peaks at 208 and 222 nm) of a high α-helical content in native enzyme (Khosro *et al*, 2001) which is similar to Amy Strplo 716. The mean residue ellipticity (MRE) was calculated using the formula given in materials and methods. The percentage of secondary structure elements was determined using the software CDPRO (Sreerama *et al*, 1999).The values of the secondary structure elements obtained were: α-helix-12%, β-sheet-33.6%, turns-21.6% and unordered-32.4%.

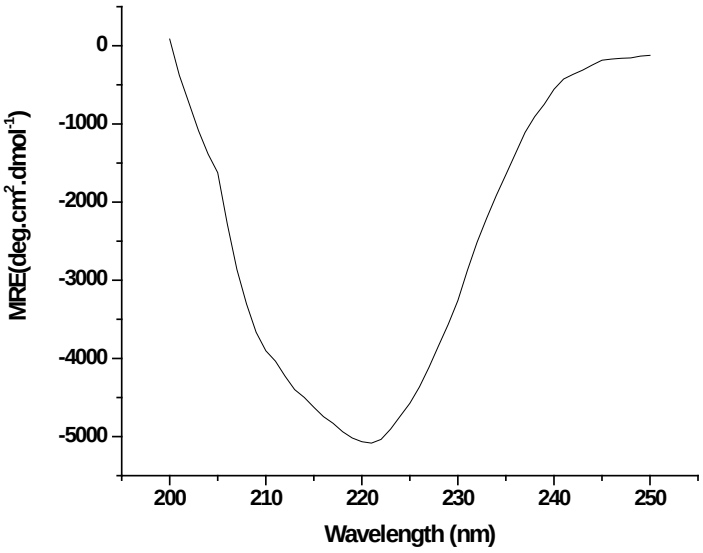

Figure 4.12*:* Far UV CD spectrum of Amy Strplo 716 (170 µg/ml) in 20 mM of Phosphate Buffer of pH 7.0

Fluorescence measurements

Fluorescence spectroscopy is widely used to study peptides and proteins. The aromatic amino acids, tryptophan, tyrosine, and phenylalanine, offer intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions. Of the three, tryptophan is the most popular probe. Tryptophan occurs as one or a few residues in most proteins and biologically active peptides.

The chemical modification studies yielded 5 tryptophan residues which are present on the surface. The native Amy Strplo 716 showed fluorescence spectrum with λ_{max} at 357 nm using excitation wavelength of 295 nm indicating trp residues in the polar environment or exposed to the solvent (Fig. 4.13). The fluorescence of proteins at 295 nm originates mainly due to tryptophan.

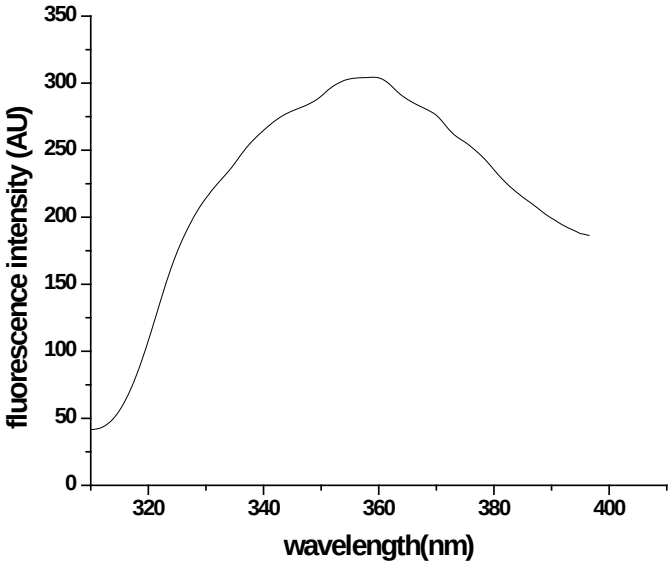

Figure 4.13: Fluorescence emission spectrum of Amy Strplo 716 (100µg/ml) in 20mM Phosphate buffer of pH 7.0 using 295 nm as excitation wavelength.

Decomposition analysis

Five spectral classes of tryptophan residues are proposed to exist in the protein (Burstein *et al*, 1973, 1977a, 1983). An extended model of discrete states (classes) was developed, assuming the existence of 5 statistically most probable classes. From the model it was suggested that following separate classes of trp residues were proposed to be probable in proteins. **(1)** Class A (λ_m=308 nm, structured spectra) comprises the fluorophores, wherein there is no formation of hydrogen-bound complexes in the excited state (exciplexes) with neighboring protein groups or solvent (Hershberger *et al*., 1981); **(2)** Class S (λ_m =316 nm, structured spectra) comprises the buried tryptophan residues which are able to form the exciplexes with 1:1 stoichiometry; **(3)** Class I (λ_m= 330–332 nm, $\Delta\lambda$=48–50 nm) represents the buried fluorophores which are able of forming the exciplexes with 2:1 stoichiometry; **(4)** Class II (λ_m= 340–342 nm, $\Delta\lambda$=53–55 nm) represents the fluorophores exposed to the bound water possessing very long dipole relaxation time preventing completion of the relaxation-induced spectral shift during the excited-state lifetime; **(5)** Class III (λ_m= 350–353 nm, $\Delta\lambda$=59–61 nm) contains rather fully exposed fluorophores surrounded by highly transportable water completely relaxing during the excitation lifetime making their spectra almost correspond with those of free aqueous tryptophan.

Decomposition analysis of the tryptophan fluorescence spectra of the native Amy Strplo 716 by PFAST (http://pfast.phys.uri.edu/pfast/) (Burstein, 2003) indicated that the tryptophan present in the native amylase belong to class III group and are in polar environment.

Solute Quenching studies of Amy Strplo 716

The quenching reaction involves physical contact between the quencher and an excited indole ring, and can be kinetically described in terms of a collisional and a static component. The most significant finding with this technique is that even tryptophan (trp) residues that are assumed to be deeply buried within globular proteins are readily quenched by the uncharged quencher acrylamide. The quenchers are able to penetrate into the matrix of globular proteins, with the penetration being facilitated by small-amplitude fluctuations in the protein structure occurring on the nanosecond time scale (Lakowicz and Weber, 1973). Ionic quenchers, being charged and heavily hydrated, should be able to quench only surface tryptophan residues (Lehrer, 1971; Burstein, 1973)

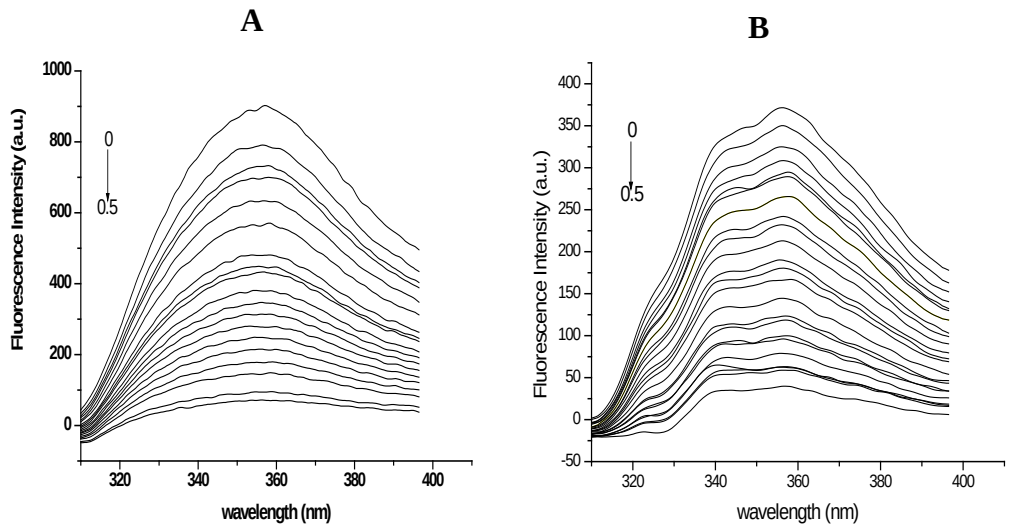

Figure 4.14: Solute quenching of Amy Strplo 716 *fluorescence* (**A**) Under native conditions quenching with acrylamide (**B**) Under denaturing conditions (treated with 6 M Gdn-HCl) with acrylamide.

Table 4.9: Extent of fluorescence quenching of Amy Strplo 716 with different quenchers.

	Quenching (%)[a]	
Quencher	***Native***	***In 6M GdnHCl***
Acrylamide (0.4 M)	***83***	***92***
Iodide ion (0.42 M)	***66***	***90***

[a] Percent quenching was calculated from the raw data.

Analysis of fluorescence quenching data

Stern-Volmer analysis of the quenching data

Among the three quenchers used, acrylamide, a neutral quencher was the most efficient showing 83 % quenching of fluorescence in presence of 0.37 M acrylamide while 66 % fluorescence was quenched by 0.42M KI. (Table 4.9).

The Stern-Volmer plot of native Amy Strplo 716 showed an upward curvature with neutral quencher acrylamide, indicating collisional and static mechanisms of quenching of the fluorescence. Collisional quenching occurs due to quenching of the fluorescence during lifetime of the excited fluorophore, while static mechanism involves complex formation between fluorophore and solute.

The Stern–Volmer plots obtained for quenching of the fluorescence with iodide ions under native conditions was linear giving K_{sv} value as and 3.9 M^{-1}. As the charged quenchers can access only surface trp, the quenching by iodide ions indicates density of positive charge around the surface trp residues. Cs^{+} did not show any effect on the fluorescence of the native or denatured protein. This may be due to the presence of positively charged residues in the vicinity of some of the exposed (or partially exposed) tryptophan residues. These repel the positively charged cesium ions, but allow the neutral acrylamide and the negatively charged iodide ion to approach the indole moieties of the tryptophan residues in their neighbourhood.

For the denatured protein (6 M GdnHCl treated), the Stern–Volmer plot still retained the upward curvature indicating collisional and static mechanisms of quenching of the fluorescence even after the change in conformation.

The 6 M GdnHCl treated protein sample showed linear Stern–Volmer plots and the K_{sv} value had substantially increased to 19.24 M^{-1} for iodide ions. This could be due

to the increased positive charge density around tryptophans due to change in conformation. These results indicate that tryptophans in Amy Strplo 716 are present in different environments; most of the residues are present on the surface and are fully exposed to solvent while only some are buried inside hydrophobic environment. Apart from the strong positive charge around the tryptophan, there could be some non-specific binding of iodide to the protein. Iodide having a large ionic radius and being negatively charged, probably binds to the positively charged amino acid residues present in the neighborhood of the single tryptophan in the protein leading to affinity quenching of the fluorescence.

These observations are in good agreement with the fluorescence spectrum of the native enzyme and also with the results of chemical modification experiments with NBS, where three tryptophan residues in α-amylase could be modified in the native enzyme and eight tryptophan residues could be modified upon denaturation of protein. This heterogeneity has also been shown by fluorescence life time measurement which is discussed later.

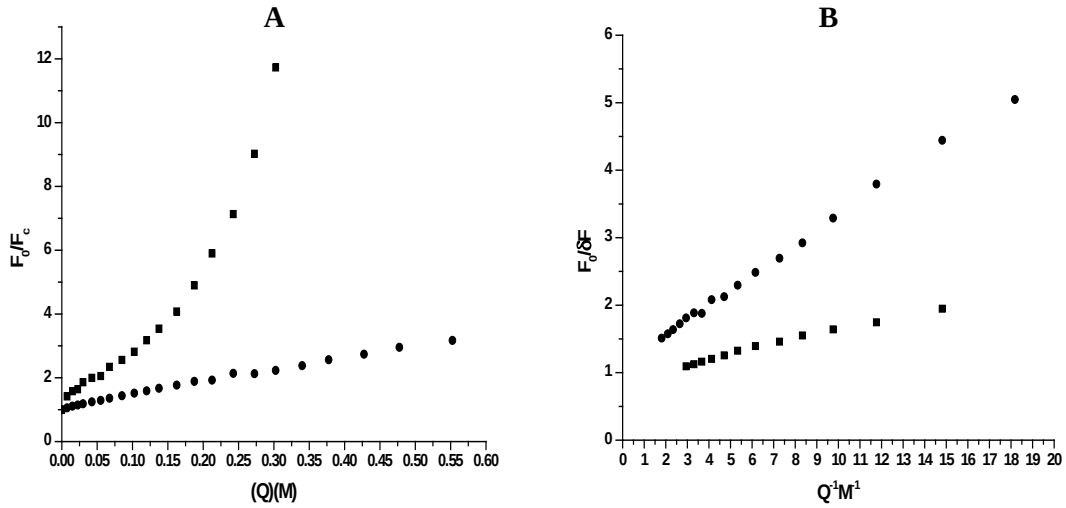

Figure 4.15: Stern–Volmer plots for the quenching of the intrinsic fluorescence of native Amy Strplo 716 with different quenchers. A; Stern-Volmer plot of native Amy Strplo 716 quenching with acrylamide (■) and KI (●). **B;** Modified Stern-Volmer plot of native Amy Strplo 716 quenching with acrylamide (■) and KI (●). Titrations were carried out as described in the "Materials and methods" section.

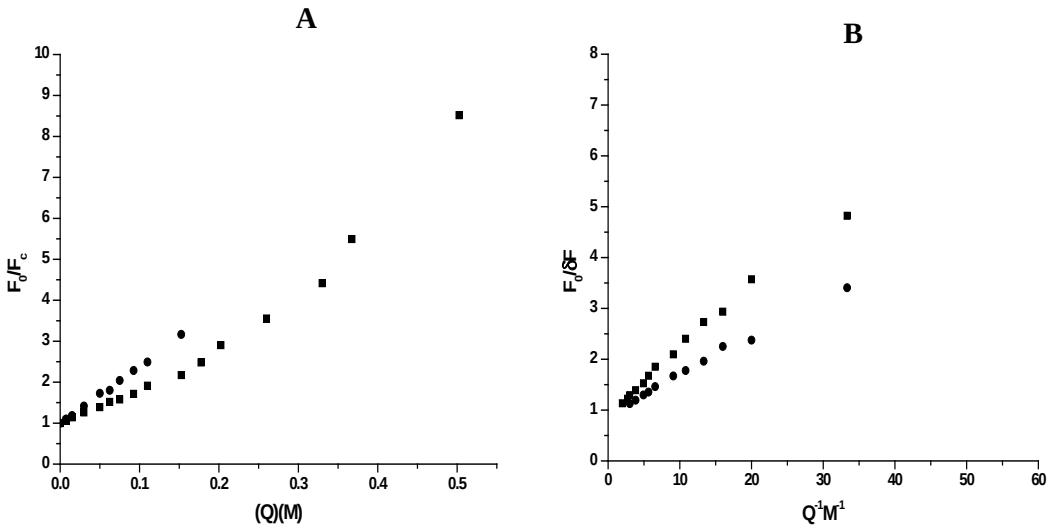

Figure 4.16: Stern-Volmer plots for the quenching of the intrinsic fluorescence of denatured Amy Strplo 716 with different quenchers. A; Stern-Volmer plot of denatured Amy Strplo 716 quenching with acrylamide (■) and KI (●). **B;** Modified Stern-Volmer plot of denatured Amy Strplo 716 quenching with acrylamide (■) and KI (●). Titrations were carried out as described in the "Materials and methods" section.

Table 4.10: **Summary of parameters obtained from the intrinsic fluorescence quenching of Amy Strplo 716 at pH 7.0.**

Quencher and condition	Ksv (M^{-1})	f_a
Acrylamide		
Native	-	1.12
Native + 6MGdnHCl	-	1.06
KI		
Native	3.9	0.65
Native+6MGdnHCl	19.24	1.25

Modified Stern-Volmer analysis of solute quenching

Modified Stern–Volmer plots obtained with the two quenchers are shown in Fig. 4.15B and 4.16B from which fa or fractional accessibility is obtained according to Eq. 2 and listed in Table 4.10. The modified Stern-Volmer plots with acrylamide and iodide are linear in native and denatured condition of the protein. Based on the fa values of the

two quenchers used, 100% and 65% of the total fluorescence was found to be accessible to acrylamide and iodide, respectively. The complete accessibility of tryptophan residues to iodide ions indicated that the protein unfolds upon treatment with 6 M Gdn-HCl.

Fluorescence lifetime measurement

The dynamics of the steady state fluorescence of Amy Strplo 716 was resolved to correlate the photo physical parameters of the protein to the structural properties. The intrinsic emission decay of the enzyme was studied in nanosecond domain (Fig. 4. 17 A) and could be described by two decay components $\tau 1$ and $\tau 2$. The corresponding relative amplitudes $\alpha 1$ and $\alpha 2$ were obtained from reconvolution fit. The decay curves obtained from the lifetime measurement of intrinsic fluorescence of Amy Strplo 716 could be fitted well into a bi-exponential curve ($\chi 2<1.112$). From these fits, two decay times $\tau 1$ (1.5314 ns) with 94.14 % contribution and $\tau 2$ (5.9697 ns) with 5.86 % contribution for the Trp fluorescence of the native enzyme were obtained, indicating the presence of more than one trp populations differentially emitting the fluorescence. In the present enzyme, the components having shorter lifetime have major contribution to the total quantum yield.

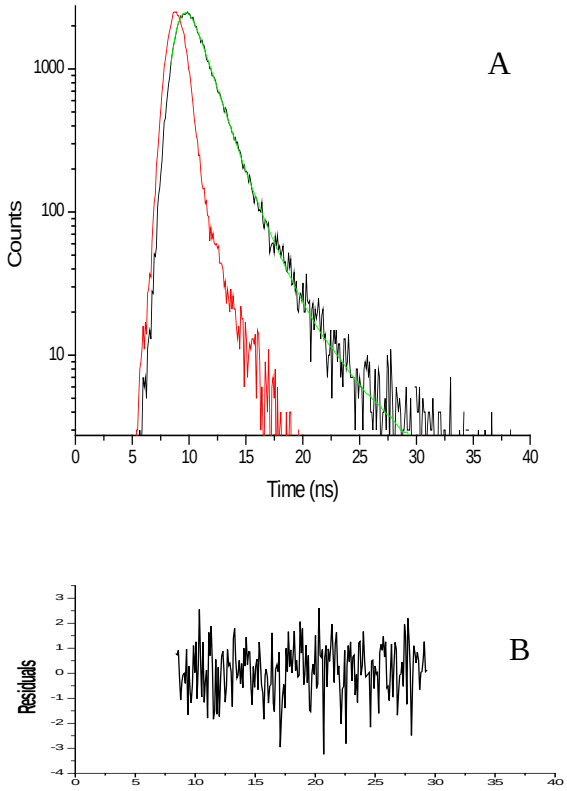

Figure 4.17: (A) Time-resolved fluorescence intensity decay of Amy Strplo 716 under native conditions. Typical fluorescence decay data obtained at 25°C using an excitation wave length of 295 nm (slit width 15 nm) and observing the fluorescence emission at 357 nm (slit width 15 nm) The protein concentration was 1.0 mg/ml in 20 mM potassium phosphate buffer of pH 7.2. The calibration time for each channel was 1.0 ns. On the Y-axis, the photon counts are presented in a logarithmic scale. The fast decaying, noisy solid line represents the IRF (instrument response function). The slower decaying line represents the experimental fluorescence decay curve. **(B)** Plot of the auto correction function of the weighted residual used to judge the goodness of fitting.

Th e λmax o f the flu decomposition analysis of steady state fluorescence spectrum has indicated class III tryptophans exposed to polar environment. Time resolved fluorescence indicates shorter lifetime exhibiting trp population with major contribution in the total fluorescence. The shorter lifetime component is supposed to be on the surface of the protein as it decays fast. Thus, the observations made in steady state and life time resolved fluorescence correlate with each other.

Effect of pH on structure of Amy Strplo 716

The enzyme showed maximum activity at pH 9.0 and is most stable in range of pH 7.0-9.0. Inactivation of the enzyme was fast below pH 5.0 and slow above pH 9.0. The fluorescence scans at different pH showed in the fluorescence intensity at pH 1, 3 and 5 as compared to pH 9.0 (Fig. 4.18A) which could be due to protonation COO^- groups on amino acid in the vicinity of tryptophan (Cowgill, 1975). ANS is a dye which has been shown to bind to hydrophobic regions in a protein and shows increased fluorescence intensity ANS binding for Amy Strplo 716 was carried out at different pH. The protein could bind ANS only at extreme acidic pH, maximum at pH 3.0 showing blue shift in the λ_{max} to 445 nm from 520 nm (Fig.4.18B). The binding of hydrophobic dye (ANS) to Amy Strplo 716 indicated the exposure of hydrophobic patches in protein at pH 3.0.

A

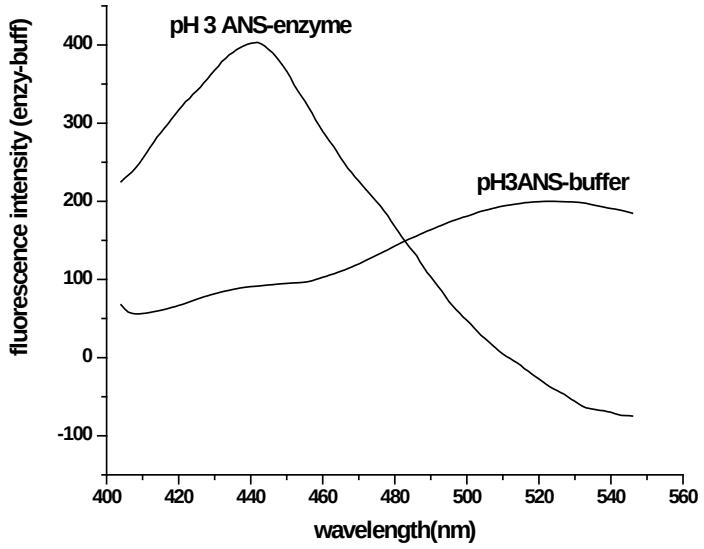

Figure 4.18: pH dependent denaturation: (A) Fluorescence spectra showing ANS binding of Amy Strplo 716 (100 µg/ml) at pH 3.0.

It is known that acids and bases disrupt the electrostatic interactions involved in protein folding. Below pH 3.0, all ionizable side chains are protonated. This leads to charge-charge repulsion resulting in protein unfolding. The side chains packing are loosened and thus hydrophobic groups are exposed to solvents i.e. the hydrophobic groups become more accessible to the solvents. Partially folded states are characterized at equilibrium under mildly denaturing conditions, such as by altering pH, addition of

salts and alcohols, chemical denaturants such as urea and guanidine hydrochloride or by changing temperature and pressure.

Far UV CD spectrum of Amy Strplo 716 (Fig 4.19 A) shows slightly rearranged and without any major loss in the secondary structure at pH 3.0. Near UV CD spectrum of Amy Strplo 716 (Fig 4.9 B) shows a altered tertiary structure at pH 3.0 especially in the region at 295 nm indicating alteration in the tryptophan environment.

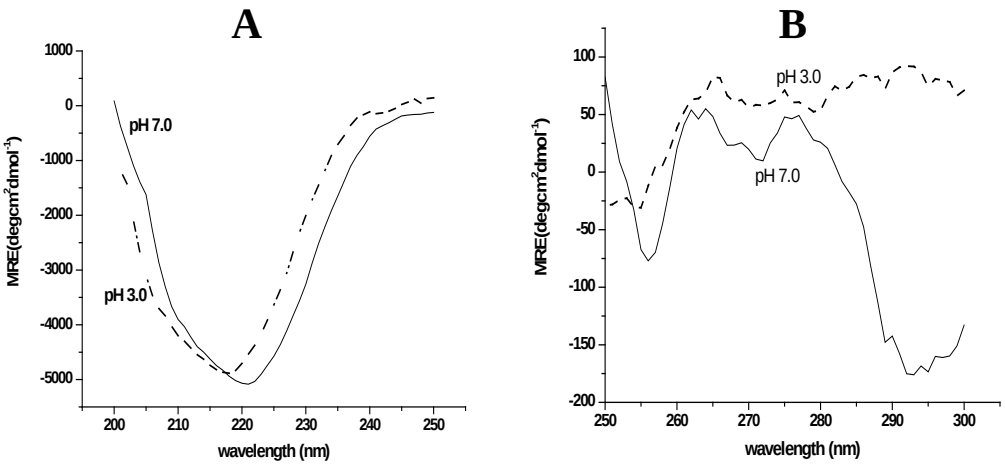

Figure 4.19: pH dependent denaturation: (A) Far Near UV CD spectrum of the native Amy Strplo 716 (170 µg/ml) at pH 7.0 and pH 3.0 **(B)** Near UV CD spectrum of the native Amy Strplo 716 and pH 3.0 Amy Strplo 716 (1 mg/ml).

The protein samples were incubated for 4h at respective pH for both the experiments. Equilibrium intermediates characterized in different proteins were found to be related to kinetic folding intermediate transiently populated in early phase of folding reaction. This partially folded state was termed ‘molten globule’ since it had shape with loosely collapsed hydrophobic core. There was formation of molten globule like intermediate in Amy Strplo 716. The protein exhibited molten-globule like intermediate with native like secondary structure and collapsed tertiary structure.

Conclusions:

1. The alkaline Amy Strplo 716 was isolated from *Streptomyces lonarensis* strain NCL 716 in potato starch media at pH 10.0. The enzyme was purified using preparative PAGE.
2. The molecular mass of Amy Strplo 716 was 37,000 Da.
3. Amy Strplo 716 may have slightly more affinity towards substrate amylose than for amylopectin and starch.
4. Active site characterization of the enzyme showed the involvement of His, Arg, Cys and Trp residues.
5. The activation energy of the enzyme was found to be 18.36 KJ mol^{-1} which is much lower than other amylases and hence Amy Strplo 716 can be of industrial importance
6. Two disulfide linkages were estimated to be present in Amy Strplo 716 . Amy Strplo 716 predominantly showed β-sheet structures.
7. Decomposition analysis of steady state fluorescence spectrum has indicated class III tryptophans, which are exposed to polar environment.
8. Acrylamide yielded an upward curvature indicating collisional and static mechanisms of quenching of fluorescence in the native as well as unfolded state.
9. The denatured Amy Strplo 716 when quenched with KI yielded higher K_{sv} value than the native indicating increase in positive charge density around the trp residue on the surface.
10. The life-time measurement of fluorescence revealed presence of more than one trp populations. In Amy Strplo 716, the components having longer lifetime have major contribution to the total fluorescence.
11. The observations made in steady state and time resolved fluorescence correlate with each other. The shorter life time component is on the surface of the protein as it decays faster.
12. There was formation of molten globule like intermediate at pH 3
13. This Amy Strplo 716 enzyme can be used as a good model protein for structure function studies and further investigation of the molecular basis of alkalophilicity.

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Chapter 5

Cloning of the gene encoding the alpha-amylase from the *Streptomyces* strain NCL 716.

Summary

This chapter describes the cloning and sequence analysis of the partial gene encoding the α-amylase from the alkalophilic *Streptomyces lonarensis* strain NCL 716 and homology modeling of the translated protein. The sequence has been submitted to the GenBank with the accession number ADZ45287. The partial gene sequence (*Amy Strplo 716*) which is 1312 bp long encodes a protein (Amy Strplo 716) of 437 amino-acids. The partial amino-acid sequence includes all the conserved regions present in other *Streptomyces* α-amylases. Homology modeling suggests that Amy Strplo 716 has 48% sequence identity with AHA ie the α-amylase from *Alteromonas haloplanctis*. The homology model of Amy Strplo 716 built using AHA as a template is ideal as it shows favourable scores for Errat, Verify3D plot and Procheck.

Introduction

The α-amylase of the alkalophilic *Streptomyces lonarensis* strain NCL 716 exhibits two important properties:

i) It is an alkaline amylase with a pH and temperature optimum of 9.0 and 45°C respectively. ii) It acts on starch to give maltotriose and maltotetraose as the major products along with maltose.

Preliminary studies carried out in this laboratory have shown that when this enzyme preparation was added as an additive to the detergent Surf, it improved the efficiency of removal of the starch based stains (Geetha, 2006). The amylase from this strain has also been bench marked with the amylases producing specific maltooligosaccharides from other *Bacillus* and *Pseudomonas* species (Table 5.1).

Table5.1 Activity of malto-oligosaccharides producing microbial α-amylases.

Organism	Enzyme produced (IU/ml)	Reference
Bacillus circulans GRS 313	98	Dey *et al*. (2003)
Bacillus sp. GM 8901	0.75	Kim *et al*. (1995)
Bacillus sp. MG-4	0.88	Takasaki *et al* (1991)
Pseudomonas sp. IMD353	29	Fogarty *et al* (1994 a & b)
Pseudomonas sp. MS300	88	Kobayashi *et al* (1998)
Pseudomonas sp.	6.5	Sakano *et al*. (1983)
Pseudomonas stutzeri MO-19	80	Nakada *et al* (1990)
Streptomyces sp.	0.85	Nakai *et al*. (1986)
Streptomyces lonarensis strain NCL 716	29.7	Present work

Hence, the above data suggest that the *Streptomyces lonarensis* strain NCL 716 is promising and could have a potential application as an additive in detergents and also for the production of G3 and G4 maltooligosaccharides.

In India, presently only four Indian Companies i.e Biocon (India) Ltd, Maps India, Advanced Biochemicals Ltd and Richore India are actively engaged in the production and handling of some industrial enzymes including amylases for detergents. At present 70% of the total enzyme requirement is being imported. Due to liberalisation of the national policy on import and export, a large number of multinational companies such as Novozymes, Genencor International, Enzyme Development Corporation, Dyadic International and Quest International have entered the country and are competing with the domestic detergent market. Indian companies such as Maps India and Advanced Biochemicals are therefore channelising their efforts to develop innovative enzymes at competitive prices.

In view of the present scenario, enzymes from alkalophiles become all the more significant. Hence, identification of strains secreting industrially useful enzymes, characterization of such enzymes and cloning the genes encoding the enzymes for developing a recombinant DNA technology to enhance enzyme production and alter enzyme properties to suit various industrial needs is relevant. Also, with emphasis shifting towards developing eco-friendly technologies to curtail environmental pollution and the need to develop innovative enzymes at competitive prices, the development of enzyme technology assumes national importance. Enzyme based technology has gained importance in western countries in view of the stringent laws which are forcing industries to shift to cleaner technologies. It is visualized that India too would have to adopt such changes in the near future if it has to be globally competitive.

As already mentioned in the earlier sections of this thesis, there is an increasing interest in the production of malto-oligosaccharides such as G3 and G4 for the food and beverage industry, because of their properties that add value to the food and health drinks. G3 and G4 are not produced by all amylases; but are produced by the enzymatic degradation of starch by only specific amylases. Currently, most of the companies have been catering to the requirements of the detergent industry. The use of G3, G4 syrup is of recent origin and Japan is presently the main producer.

Detailed enzyme characterization, cloning of the gene encoding the α-amylase and study of the enzyme crystal structure are primary requirements for developing a recombinant DNA technology for this alkaline α-amylase from the *Streptomyces lonarensis* strain NCL 716. Knowledge of the primary structure of the gene is required to carry out any site directed mutagenesis work to alter the enzyme properties to suit different industrial needs.

The genes encoding the maltotetraose producing amylase have been cloned from *Pseudomonas stutzeri* (Fujita *et al*., 1989, 1990), *Pseudomonas sp.* KFCC 10818 (Kang *et al*, 2001), *Pseudomonas saccharophila* (Zhou *et al*, 1989) and *Streptomyces albus* KSM-35 (Lee *et al*, 1996 a & b; Min *et al*, 1998). Takasaki, US Patent Application No. 4,925,795 (1986) describes a method of using G-4 amylase to produce high maltotetraose and maltose content starch hydrolysates. US Patent 5958749 (Kubota *et al*, 1999) describes the cloning of the gene encoding a maltotetraose forming amylase. Novozymes A/S (2000) Patent WO/2000/059307 (Nielsen and Schaefer, 2000) describes the preparation of dough and baked products using an amylase which hydrolyses starch to form maltotriose which helps to retard staling of bread US Patent 4,113,509 (Leach *et al*, 1978) describes the enzymatic preparation of maltose-maltotriose starch hydrolysates.

The gene encoding the maltotriose producing amylase has been cloned from *Bacillus subtilis* SUH4-2 (Min *et al*, 1998), *Streptococcus bovis* 148 (Satoh *et al*, 1997), *Thermobifida fusca* (Yang and Liu, 2004), *Brachybacterium sp.* LB25 (Doukyu *et al*, 2008), *Natronococcus sp* (Kobayashi *et al*, 1994) and *Sclerotinia sclerotiorum* (Imen *et al*, 2008). The genes of maltotetraose producing amylase (G4-amylase) from *Pseudomonas saccharophila* (Zhou, 1989), maltopentaose producing amylase (G5-amylase) from *Pseudomonas* sp (Shida, 1982) and G6 amylase from *Bacillus* sp (Tsukamota, 1989) have been cloned and their nucleotide sequences have been reported. There exists an amino acid residue difference in these regions between *K.pneumoniae* G6 amylase and MAlS of *E.coli*.

This chapter describes the cloning of the partial gene encoding the α-amylase from the alkalophilic *Streptomyces lonarensis* strain NCL 716, as a first step towards developing a recombinant DNA technology for this enzyme.

Materials and Methods

Materials

Following chemicals, enzymes etc. were purchased from various suppliers such as Merck, SRL, Hi-Media, Sigma Chemical Company, Qualigens, Bangalore Genei, Promega and Invitrogen.

Merck, SRL, and Sisco Chemical India:

NaCl, KCl, $MgCl_2$, $ZnCl_2$, $CoCl_2$, $CaCl_2$, $AlCl_3$, $HgCl_2$, $CuSO_4$, $FeSO_4$, Glycerol, Acetic acid, phenol, Chloroform, Iso-amylalcohol, HCl, NaOH, Agarose, Formamide, Ethanol, Abosolute ethanol , Tris-HCl , EDTA, Trisodium citrate, Agarose, etc.

Hi-media

Bacto yeast extract, Bacto tryptone, Yeast extract, Agar, Yeast extract, Malt extract, Peptone, D-glucose, etc.

Sigma Chemical Company:

Dinitro salicylic acid (DNSA) , Ethidium bromide, Dimethylsulfoxide (DMSO), bis-acrylamide, Tween 20, Tween 80, SDS, Triton X-100 etc.

Qualigens Fine Chemicals:

Sodium hydroxide, Sodium carbonate, MgCl2.

Banglore Genei limited:

Taq polymerase, Taq buffer with $MgCl_2$, 10mM dNTPs, 5X loading dye and various restriction enzymes (see list in appendix), Marker DNA ladder (see list in appendix).

Lonza : SeaKem LE agarose

Invitrogen : SNAP columns, glycogen, pCR4 TOPO cloning kit which included the vector ans *E.coli* TOP 10 cells.

New England Biolabs : Quick Ligation kit.

In addition, various kits such as DNA Isolation Kit from USB corporation, USA; PCR Purification Kit, Qiagen Ltd, Gel Extraction Kit from Qiagen Ltd and DNA Ligation Kit (Fermentas Ltd) were also usedin the course of this work.

Plastic ware

Following plastic was procured from Tarsons, Axygen and Laxbro (India).

Disposable Petri dishes, Eppendorf tubes, PCR vial, micro-pipette tips, centrifuge tubes, micropipettes.

Glassware

Following glassware were purchased from Borosil or Corning Ltd (India).

Toothpicks,Test tubes, glass-beakers, spreader, conical flasks, thermometer and measuring cylinder.

Instruments

Following instruments were to carry out daily experiments:

Shaker-incubator, magnetic stirrer, thermal cycler (MJ Research PTC-100), Spectrophotometer (Shimadzu) pH meter, laminar air flow, 20°C and -70°C freezers, heating block, electrophoresis apparatus, Gel Documentation System (Syngene), centrifuge (Refrigerated REMI CPR 24), microwave oven, water bath (thermostat), ice machine, UV lamp, gas burner, power pack.

Media Component and Preparation

Luria Bertani broth (LB):

This media has been used for all the molecular biology work.

Per 1000 ml:

Tryptone	10 g
Sodium Chloride	10 g
Yeast Extract	5 g

The pH is adjusted to 7.2 with 0.1M Sodium Hydroxide (NaOH). The final volume is made up to 1000 ml with double distilled water. The medium is autoclaved at 15 lb for 20 min.

LB agar:

1.2% of bacterial grade agar is added to LB medium autoclaved at 15 lb for 20 min.

Ampicillin is added to an effective concentration of 100 mg/ml after cooling the medium to 45°C.

SOC medium:

The following components were added to 900 ml of distilled H_2O

Bacto Tryptone	20 g
Bacto Yeast Extract	5 g
5M NaCl	2 ml
1M KCl	2.5ml
1M $MgCl_2$	10 ml
1M $MgSO_4$	10 ml
1M glucose	20 ml

The pH was adjusted to 7.0 after which the volume was adjusted to 1L with distilled H_2O. The medium was autoclaved at 15 lb for 20 min. Note: Dissolve every component except magnesium salts and glucose. These two components should be filter-sterilised and added to the autoclaved medium.

MGYP Medium:

Malt Extract	0.3%
Glucose	1%
Yeast Extract	0.5%
Peptone	0.5%
K_2HPO_4 (5%)	2ml
$MgSO_4$ (2%)	1ml

5 ml of 10% sodium carbonate was added to 50 ml medium (separately autoclaved and added just before inoculation into media).

Buffers/Solutions

Polysaccharide Eliminating Buffer (for DNA extraction by Wang *et al*, 2005)

20% Ethanol

0.5M Potassium Acetate

Lysis Buffer: (for DNA extraction by Wang *et al*, 2005)

0.1M Tris HCl (pH 7.5)

1.5% SDS

500 mM NaCl

10 mM 2-mercaptoethanol

1%w/V PVP

CTAB-NaCl mix /1 ml (for DNA extraction by Dellaporta *et al*, 1983)

NaCl	0.7 M
CTAB	0.1 g

0.7 M NaCl was heated to 55°C and CTAB was added to it to dissolve.

TE25S Buffer

Composition for 5 ml

Tris HCl (1M, pH 8.0)	0.125 ml
EDTA (25 mM, pH 8.0)	0.5 ml
Sucrose	0.5134 g

NEB buffer (50X):

For 500 ml

Tris	121 g
Glacial acetic acid	28.54 g
EDTA	18.6 g

EDTA was dissolved in a boiling water bath or in a microwave oven. Then tris was added and dissolved. Glacial acetic acid was then added and pH was checked and adjusted to 7.9-8.2.with NaOH and CH_3COOH.

$T_{10}E_1$ buffer

Tris-HCl buffer (1M, pH 8.0)	1 ml
EDTA (0.5M)	0.2 ml

Make the volume to 100 ml with double distilled water and autoclave at 15 lb for 20 min.

0.1 M $CaCl_2$ solution

1.1098 mg of $CaCl_2$ was dissolved in 100 ml distilled water and autoclaved at 15 lb for 20 min.

Preparation of Solutions for plasmid prep:

Solution I: 50 mM glucose, 25 mM Tris-HCl (pH 8), 10 mM EDTA

Solution II: 0.2M Sodium Hydroxide (NaOH), 1% SDS

Solution III: 5M Potassium acetate (60ml), Glacial acetic acid (11.5 ml in 28.5 ml water)

Extraction buffer: (for DNA extraction by Wang *et al*, 2005)

1M Tris	0.2 ml
EDTA	80 µl
Sorbitol	0.26 g
BSA	0.004 g
Beta-mercaptoethanol	4 ml
Polyethylene glycol	0.4 g
N-lauryl sarcosine	0.04 g

Make up volume to 4 ml with sterile double distilled water.

2X Kirby mix: (for 1 ml)

SDS	0.02 g

Sodium 4 amino salicilate	0.12 g
2 M Tris HCl (pH 8)	50 µl
Equilibrated phenol	60 µl

Dissolve SDS and sodium 4 amino salicilate in 890 µl water. Add 50 µl Tris HCl buffer and phenol 60 µl. Do not autoclave and store away from light at 4°C.

STSE buffer (Kieser *et al*, 2000)

Component	**Final Concentration**
Sucrose	10.3 %
Tris HCl	25 mM
EDTA	25 mM
RNase A	100 ng/ml
Lysozyme	3 mg/ml
Adjust pH 8.0	

(Add RNase and lysozyme just before use)

Prehybridizarion mix:	**(50 ml)**
NaCl	2.2912 g
Tri-Sodium Citrate hydrate	1.8387 g
Denhardt Solution (final conc. 5%)	8.3 ml
10% SDS	3 ml
30% Dextran sulphate	2.5 ml
0.25M EDTA	0.5 ml
1M $NaPO_4$	2.5 ml

Denhardt Solution:	**(10 ml)**
Ficoll	0.2 g

PVP	0.2 g
BSA	0.2 g

Dissolve the components in minimum 5-6 ml double distilled water and make up volume to 10 ml. Add 360 µl of salmon sperm DNA to 50 ml pre-hybridization buffer before use.

Note: Salmon sperm DNA aliquot was boiled for 10 minute to make it ssDNA

Methods

1. All microbiological procedures were done in sterile conditions in laminar air flow.

2. All biochemical additions were made at appropriate temperature or conditions as required.

Production of alkalophilic Streptomyces NCL strain biomass

1. MGYP broth was prepared and inoculated with the 7 day old culture of *Streptomyces lonarensis* strain NCL 716.

2. Flasks were kept on shaker at 110 rpm, 28 °C and harvested at 3-4 days.

3. The culture filtrate was centrifuged in a Remi-Centrifuge at 4 °C, 10,000 rpm for 15 min.

4. The supernatant was discarded and the biomass (pellet) was stored at -70 °C for future use.

***Isolation of genomic DNA from the Streptomyces strain* NCL 716.**

Six protocols as described below were used to isolate the genomic DNA.

Protocol 1

DNA was isolated by the protocol described by Kirby *et al*, 1967.

1. 15 g to 18 g cell mass was taken.
2. With glass beads this cell mass was dispersed gently and 100 ml of 1% (w/v) sodium tri-isopropylnaphthalene sulphonate and 6% (w/v) sodium 4-aminosalicylate was added. To each 100 ml, 3 ml of phenol-cresol mixture was added.
3. 200 ml of the same mixture was added and the viscosity increased when the mixture was shaken by hand in a stoppered flask.

4. 300 ml of the phenol-cresol mixture was added and the mixture was again shaken by hand until thoroughly mixed and then by a mechanical shaker for a further 20 min at 20°C.
5. The mix was centrifuged at 12,000 g for 20 min at 5°C to separate phases.
6. Top layer was carefully poured off, made 3% with respect to NaCl and mixed with 0.5 volume of phenol-cresol mixture (10min at 20°C).
7. The phases were again separated by centrifugation (17,000 g for 10 min at 5°C).
8. Aqueous phase was carefully poured off, made 20% with respect to sodium benzoate (when solution becomes somewhat less opalescent).
9. DNA was precipitated by careful addition of m-cresol (0.1-0.12 vol)
10. Viscosity of the mixture increases during the addition and then falls when the DNA suddenly coagulates. Coagulation is difficult to see and is best observed by pouring the mixture.
11. After centrifugation (700 g for 10 min, at 5°C), the DNA separates as a gel that partly sediments and partly floats on the surface.
12. Total gel is combined and mixed with 0.1M sodium acetate buffer, pH 6.0 (15 ml).
13. Solution is best obtained by careful shaking, centrifuging off the undissolved gel (500 g for 2min), pouring off the supernatant solution and adding 0.1M sodium acetate buffer; pH 6.0 (5-10ml) to gel.
14. Process was repeated until the DNA was completely dissolved.
15. Solution was then made 3% with respect to NaCl and 20% with respect to sodium benzoate and DNA precipitated with m-cresol (0.15 - 0.18 vol; as n phenol was present in the mixture).
16. Solution of DNA (in 0.1M sodium acetate buffer, pH 6.0) was made 4M with respect to NaCl and solution stored at 0°C for 16 h, during this time rRNA is precipitated.
17. Precipitate is removed by careful layering over 6M-NaBr (4ml) and then centrifuged (80,000 g for 1h at 5°C)
18. Centrifugation of the RNA from the 4M NaCl through 6M-NaBr was essential to obtain good separation. From the viscous solution of DNA that floats while the RNA sediments to the bottom.
19. Clear DNA solution is poured off and dialyzed against 0.1M sodium acetate buffer, pH 6.0 for 5mM KF ($KF.2H_2O$).
20. DNA yield was estimated from the E_{260} value.

Protocol 2

DNA was isolated using the PrepEase® Genomic DNA isolation kit from USB Corporation, USA, as follows:

1. About 25 mg of bacterial biomass was homogenized in 0.24 ml of homogenization buffer until the sample was completely lysed. (by vortexing)
2. 0.2 ml of chloroform: isoamyl alcohol (24:1) and 0.8ml of protein precipitation buffer were added to the lysate and vortexed (10 pulses at full speed). The sample was centrifuged at 13000 g for 4 min at RT.
3. 0.88 ml of upper aqueous phase was transferred to a clean eppendorf containing 0.62 ml of isopropanol. The solution was mixed by inverting the tube at least 15 times and then spun at 13000 g for 4 min at 20°C.
4. The supernatant was discarded without disturbing the pellet and the pellet washed once with 1 ml 70% ethanol by vortexing. It was then centrifuged at 13000 g for 2 min.
5. The supernatant was discarded and the pellet was allowed to dry at room temperature.
6. The DNA pellet was resuspended by vortexing in 50 to 300 µl of DNA resuspension buffer and the DNA pellet was allowed to dissolve by keeping it at 4°C).
7. RNase A was added to a final concentration of 50 µg/ml and incubated at 37°C for 1 h.
8. The solution was deproteinized by adding equal volume of phenol:chloroform : isoamyl alcohol was added (i.e. $CHCl_3$: IAA and tris equilibrated phenol in the ratio of 1:1) and centrifuged at 10,000 rpm for 10 min at10°C.
9. The aqueous phase was taken in a fresh eppendorf and to that equal volume of $CHCl_3$**:** IAA was added and the solution was centrifuged at 10,000 rpm for 10 min at 10°C.
10. The aqueous phase was transferred to another eppendorf vial and $1/10^{th}$ volume of 3 M sodium acetate and double volume of chilled ethanol was added. This solution was kept at -20°C overnight for the DNA to precipitate.

11. The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at Centrifuged at 10,000 rpm for 10 min at 10°C.

12. To the pellet, 500 µl of 70% ethanol was added and vortexed followed by centrifugation at 10,000 rpm for 2 min at 10°C. The DNA pellet was dried at room temperature for about 15 min till there was no trace of alcohol.

13. Then DNA pellet was dissolved in $T_{10}E_1$ (pH 7.4) and used for further experiments.

Protocol 3

Genomic DNA isolation by CTAB method (Dellaporta *et al*, 1983)

1. 0.5 g cell mass was weighed in a 2 ml eppendorf vial and washed twice with autoclaved double distilled water.

2. The pellet was resuspended in 0.5 ml TE25S buffer.

3. 10 µl lysozyme solution (100mg/ml) was added to it and incubated at 37°C for 1 h.
4. 5 µl proteinase K solution (20mg/ml) and 30 µl of 10% SDS were added to it and the solution was mixed gently by inversion and then incubated at 55°C for 1h. It was gently mixed occasionally.

5. 100 µl of l 5M NaCl was added in it and mixed thoroughly by inversion.

6. To it 65 µl of CTAB-NaCl mix was added and the solution was mixed thoroughly and incubated at 55°C for 10 min.

7. This sample was cooled to 37°C and to that 500 µl chloroform:isoamylalcohol mix (24:1) was added. The suspension was mixed by inversion for 30 min and then centrifuged at 10,000 rpm for 15 min at 20°C.

8. The supernatant was transferred to a fresh tube and 0.6 volumes of iso-propyl alcohol was added and mixed by gentle inversion.

9. The DNA was pelleted by centrifuging it at 5000 rpm for 10 min at room temperature.

10. The supernatant was discarded and the pellet was washed briefly with 70% ethanol by vortexing, followed by centrifuging at 5000 rpm for 10 min at room temperature.

11. The DNA pellet was air dried and resuspended in 50-100 µl of $T_{10}E_1$ (pH 8).

Protocol 4

Genomic DNA isolation by the protocol of Crowley et al (2003).

1. 1 g cell mass was ground to a fine powder in a pestle and mortar using liquid nitrogen.
1. 4 ml of extraction buffer was added to it and the mix was allowed to thaw.
2. 5 ml of 4 M NaCl and 1ml 10% (w/v) CTAB were added and the mix was incubated at 60°C for 1 h.
3. The mix was then centrifuged at 13,793 g for 10 min and the supernatant was
4. Transferred to sterile tubes. 1 volume of chloroform:octanol (24:1, v/v) was added
5. To the supernatant and centrifuged at 13,793 g for 10min.
6. The aqueous layer was carefully aspirated and to that 2 volumes of ice-cold IPA (0°C) and NaCl was added to a final concentration of 2M NaCl and stored at 0°C for 2 h to precipitate the genomic DNA. (Note: Add IPA and NaCl immediately one after the other).
7. The above mix was centrifuged at 13,793 g for 5 min.
8. The pellet was washed with 70% ethanol and air dried.
9. 100 µl $T_{10}E_1$ (pH 7.4) was added to the dried pellet. 2 µl RNase was added to it to a final concentration of 50µg/ml and incubated at 37°C for an hour followed by de-proteinisation with chloroform:isoamyl alcohol. The pellet was washed with 70% ethanol followed by centrifugation at 10,000 rpm for 10 min at 10^0C.
10. The pellet was air dried and finally dissolved in $T_{10}E_1$ (pH 7.4) and used for further experiments.

Protocol 5

Genomic DNA isolation by the protocol of Kieser et al (2000)

1. 1 g cell mass was weighed and washed with 1 ml STSE.
2. The washed cell mass was resuspended by vortexing in 500 µl TSE buffer having lysozyme (3mg/ml) and RNase A (100 ng/ml).
3. The sample was incubated at 37°C for 15 min.

4. 400 µl of 2X Kirby mix was added and vortexed vigorously for 1 min. 800 µl phenol: chloroform: IAA was added in that and vortexed vigorously for 15 sec. followed by centrifugation at 17000 g for 10 min at 4°C.

5. The supernatant was transferred to a fresh eppendorf and to that 70 µl unbuffered 3M sodium acetate and 300 µl phenol: chloroform: IAA were added. The solution was vortexed vigorously for 1 min.

6. The aqueous phase was transferred to another eppendorf and 0.7 volume of IPA was added to it. This solution was centrifuged at 20,000 g for 30 min at 4°C.

7. The pellet was washed with 70% ethanol and air dried and dissolved in $T_{10}E_1$ (pH 8.0).

Protocol 6

Genomic DNA extraction by the protocol of Wang et al (2005).

1. 0.5 g of cell mass was weighed in a 2 ml eppendorf and 1 ml of polysaccharide eliminating buffer was added to it. The suspension was vortexed and centrifuged 4000 rpm for 1 min at room temperature.

2. The supernatant was discarded and the pellet was washed twice with polysaccharide eliminating buffer.

3. The cell pellet was then ground to a fine powder with liquid nitrogen.

4. Lysis buffer was added in that (1ml lysis buffer added per 0.2g cell mass) and the suspension was incubated at 65°C for 1 h.

5. 333 µl of potassium acetate (5M) was added per ml of this lysis mixture. This was mixed gently and immediately kept in ice for 30 min. It was then centrifuged at 15000 g for 30 min at room temperature.

6. The supernatant was carefully aspirated and transferred to a fresh vial. Equal volume of $CHCl_3$:IAA was added and the mix was gently mixed by inversion and centrifuged at 10,600 g for 10 min at 20^{o}C.

7. Equal volume of isopropyl alcohol was added to the aqueous layer and mixed by inverting the vial, followed by centrifugation at 10,600 g for 10 min at 20^{o}C.

8. The supernatant was discarded and the DNA pellet was washed with 70% ethanol by vortexing followed by centrifugation at 10,000 rpm 2 min at 10°C.

9. The DNA pellet was air dried and dissolved in $T_{10}E_1$ (pH 8).

10. The DNA solution was given RNase treatment and the DNA pellet was dissolved in $T_{10}E_1$ for further experiments.

Estimation of concentration and quality of DNA samples by gel electrophoresis

An aliquot of the DNA sample was mixed with 5X loading buffer and loaded on a 0.8% agarose gel containing ethidium bromide (EtBr-10 mg/ml). Electrophoresis was carried out in 0.5X NEB buffer for 20 minutes at 160 mV using λ-HindIII /medium range molecular weight marker as a reference. Resolved DNA bands were visualized under UV-light and documented by photography. The concentration of DNA samples was determined by comparing the intensity of fluorescence with commercial preparations of λ DNA of known concentration.

Restriction enzyme digestion

Genomic DNA or vector DNA was digested with the appropriate restriction enzyme according to the manufacturer's instructions. 1 µg DNA was digested with 3-5 units of enzyme using the appropriate buffer. Care was taken that the concentration of RE should not exceed 10% of final volume of the reaction mixture. A typical composition of the restriction enzyme digestion reaction mixture was as follows:

Genomic / vector DNA	X ul
10X buffer	10% of total
Restriction enzyme (10 U/µl)	Y µl (final concentration 3-5 U)
Sterile water	to a final volume of Z µl.
Total volume	Z µl

An aliquot of it was loaded on 0.8% agarose gel to confirm complete digestion of the DNA.

The digest was purified as follows: To the digest equal volume of phenol-chloroform-isoamyl alcohol was added. The mixture was vortexed briefly and centrifuged at 10,000 rpm for 10 min. Equal volume of chloroform-isoamyl alcohol was added to the aqueous layer and the mixture was vortexed briefly followed by centrifugation at 10,000 rpm for 10 min. The DNA was precipitated from the aqueous layer by addition of 1/10th volume

of 3M sodium acetate and twice the volume of chilled ethanol. When the amount of DNA to be precipitated was very low, 0.2 µg/ul glycogen was added as a carrier. Precipitation was done by keeping the above solution at -20°C overnight. The DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, air dried and dissolved it in $T_{10}E_1$.

Elution of DNA fragments from low melting point (LMP) agarose gel using the QIAGEN Gel Extraction Kit (Cat #28704)

1. The portion of the gel containing the DNA fragments of interest was cut out using a sterile scalpel and was weighed. .
2. 300 µl of buffer QG was added per 100 mg of agarose piece and was incubated in a water bath at 50°C for 10 min. The contents were mixed by inverting the tube 2–3 times during incubation.
3. After the gel piece dissolved completely, 100 After gel piece dissolved completely, 100 After gel piece dissolved completely check colour of mixture is yellow). 100 µl of isopropanol was added per 100 mg gel and the contents mixed by inverting the vial.
4. A QIA quick spin column was placed in a 2 ml collection tube that was provided in the kit and 700 µl sample was added to the spin column and spun at 10,000 g at 1 min at 25°C.
5. The flow through was discarded and the QIA quick column was put back in the same collection tube. (Collection tubes are reused to reduce plastic waste).
6. Steps 4 and 5 were repeated till all of the solution was passed through the column.
7. The empty column was spun for 1min at 10,000 g for 25°C.
8. DNA was eluted from the column by passing 50 µl of buffer EB through the spin column. The eluent was collected and the procedure was repeated two more times.
9. The concentration of the DNA in the eluents was determined by agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The composition of a typical PCR reaction mix was as follows:

DNA (template 25-50 µg):	x µl
10X PCR buffer:	2.5 µl
1 mM dNTPs :	2.5 µl
100 pmoles/ml forward primer:	1.0 µl
100 pmoles / ml reverse primer:	1.0 µl
Taq Polymerase (3U/ml):	0.25 µl (0.75 U)
Sterile water to a final volume of	25 µl

A master mix/cocktail was made when additions had to be made in more than one vial. The cocktail contained all components of the PCR reaction. The cocktail was dispensed in individual vials and the template/primer was dispensed in individual vials according to the requirement. The annealing temperature was decided according to the Tm of the primer pairs used. DMSO was used as additive in PCR reaction for implication of the template.

The following PCR program was used:

(I) Initial denaturation: 5 min.

(II) 25 cycles each of

a) Denaturation : 30 sec

b) annealing : 45 sec

c) extension : 1 min

d) Final extension: 5 min.

For cloning a PCR product, a final extension of 20 min was given.

Elution of PCR product from gel

The portion of the agarose gel containing the DNA fragment of interest was cut with a clean scalpel and transferred to a SNAP column and centrifuged at 10,000 rpm for 2 min at 25^{o}C. The eluted sample contains the DNA fragment. Its concentration was determined by agarose gel electrophoresis.

Cloning of the PCR product into pCR 4 TOPO vector

PCR products were cloned into pCR4 TOPO (Invitrogen) according to the manufacturer's instructions.

A typical cloning reaction had the following components:

Vector conc. (10 ng/µl) 0.5 µl.

DNA fragment (5-20 ng)	3-4 µl
Salt solution	1.0 µl

Sterile water was added to make the final volume of 6.0 µl

The above reaction was incubated at 22^0C in a PCR machine for 1 h.

An aliquot 3-4 µl was used for each transformation experiment.

Transformation of host E. coli TOP 10 Cells

Preparation of competent cells

All steps were carried out under sterile conditions in a laminar flow.

1. A glycerol stock of *E. coli TOP 10 E.coli cells* was inoculated into 10 ml of LB broth and grown at 37°C overnight in an incubation shaker.
2. 1% of this overnight grown culture was inoculated into 50 ml of LB broth and allowed to grow at 37°C with shaking, till an OD of 0.45-0.55 was attained.
3. Optical density (O.D.) of the culture was checked at 600 nm against a blank of LB broth.
4. The culture was immediately chilled on ice and the cells were harvested by centrifuging the culture at 6000 rpm for 10 min at 10°C.
5. The supernatant was discarded and the cell pellet was resuspended in 10 ml of 0.1M $CaCl_2$ & was kept in ice for half an hour.
6. It was then centrifuged at 6000 rpm for 10 min at 10°C. The supernatant was discarded and the cell pellet was resuspended in 1 ml of 0.1M $CaCl_2$ and kept in ice overnight for the cells to become competent.

Transformation of host cells

1. 100 µl of competent cells were dispensed in 1.5 ml vials.
2. 4 µl of the TOPO cloning reaction mix was added to the cells and the cells were kept in ice for half an hour.
3. The cells were then subjected to heat shock for 2 min at 42°C followed by chilling on ice for 10 min. 250 µl of SOC medium was added and the vials were shaken at 37°C for 1 h.

4. The transformed mix was plated out on LB plates containing ampicillin. Positive (LB) and negative controls (LB+ampicillin) were also made.

5. Plates were kept for overnight incubation at 37^{o}C. Colonies were transferred to master plates and these plates were preserved for further analysis.

Preparation of Master Plates

1. Recombinants were transferred to master plates with the help of sterile toothpicks. Square grids of approximately 0.5 cm were marked with a marker at the bottom of the plate. The colonies were transferred from the original plate to master plate using sterile tooth picks. One colony was transferred per square.
2. Simultaneously, inoculations for bacterial supernatant are also made. Pools of five colonies were inoculated into a single vial of 500 µl for preparation of bacterial supernatants.
3. Vials and plates were incubated at 37°C.
4. Preparation of bacterial supernatants by freeze-thaw lysis method.
5. The cells were allowed to grow overnight at 37˚C with shaking at 170 rpm.
6. Overnight grown culture was centrifuged at 8000 rpm for 10 min at 4˚C. The supernatant was discarded and the cell pellet was suspended in 100 µl of sterile water and vortexed.
7. The pellet was kept at 90-95˚C for 2 min.
8. Then, the vial was transferred to -70˚C for 10 minutes and this cycle of freezing and thawing was repeated thrice.
9. Finally the solution in the vials was centrifuged at 8000 rpm for 5 min at 4°C. The pellet was discarded and the bacterial supernatant was used for PCR.

PCR of the bacterial supernatants

1. The PCR of pools was carried out using forward and reverse primers. 5 µl of the bacterial supernatant containing DNA was used as a template.
2. The PCR conditions were the same as described earlier in this section.
3. The positive pools were identified by the presence of the insert band.

4. To identify the positive colony from the positive pool, bacterial supernatants of individual colonies of the positive pools were made as described above and the PCR was carried out using the same pair of primers to identify the positive clone.

Plasmid Preparation by Alkali Lysis Method

Plasmids of positive clones were isolated by the alkaline lysis method (Maniatis *et al*, 1982)

Requirements: Solution I, LB broth, 70% ethanol, Solution II, Ampicillin, $T_{10}E_1$ buffer, Solution III, Ice cold ethanol, Chloroform-isoamyl alcohol (24:1) mix, phenol-chloroform-isoamyl mix.

Procedure:

1. Single colony or a glycerol stock of the clone was inoculated into 10 ml of Luria-Bertani (LB) medium containing 100 µg/ml of ampicillin, and the culture was allowed to grow at 37˚C overnight at 200 rpm.
2. The cells were harvested by centrifuging at 8000 rpm for 10 min at 4^oC.
3. The cell pellet was re-suspended in 10 µl of solution I and vortexed for few seconds.
4. 200 µl of freshly prepared solution II was added and left for 10 min at room temperature.
5. 150 µl of ice-cold solution III was added to each vial and left on ice for 15 min.
6. Vials were centrifuged at 8,000 rpm for 10 min. The supernatant was transferred to fresh tubes and equal volume of phenol - CHCL3: IAA (1:1 by volume) was added to the supernatant. .
7. The mixture was centrifuged at 8,000 rpm for 10 min at 4˚C. The upper aqueous layer was transferred to another vial and equal volume of chloroform: isoamyl-alcohol mixture (24: 1 by volume) was added to it and the mixture was centrifuged at 8000 rpm for 10 min at 4˚C.
8. The upper aqueous layer was then treated with RNase (50 µg/ml) at 37 ˚C for 1 h. Equal volume of chloroform – IAA was added to the RNase treated aqueous layer for denaturing the RNase and the solution was centrifuged at 10,000 rpm for 10 min. at 4˚C.

9. The upper aqueous layer was transferred to a fresh vial and 1.5- 2 volumes of chilled ethanol was added to it. Each sample was gently mixed by inverting the tube and the DNA was allowed to precipitate by keeping the vials at – 20 ˚C overnight.
10. The DNA was then collected by centrifugation at 8000 rpm for 10 min. at 4 ˚C. The DNA pellet was washed once with 70% alcohol, dried in a vacuum speed drier and dissolved in a minimum volume of T_{10} E_1 buffer (pH 7.5).
11. Concentration of plasmid prepared was checked on a 0.8 % agarose gel.

Preparation of Glycerol Stocks

Glycerol stocks of host strain and positive clones were prepared by mixing 140 µl of overnight grown culture with 60 µl of 50% glycerol and stored at -70^{o}C.

Sequencing of plasmid or PCR product

The plasmid DNAs of the positive clones was sent for sequencing to Genome Biotech Ltd, Pune. Sequencing was done by using dideoxy chain termination automated sequencing method and analyzed by using fluorescent labels.

Southern Blotting

The Southern blot was prepared using the alkaline transfer protocol (Maniatis 1982) as follows:

1. Agarose gel (0.8%) was prepared containing ethidium bromide. 2.5µl (125 ng) of DNA molecular weight marker, DNA digest and the probe fragment (unlabelled- for control) were loaded onto the gel. The gel was electrophoresed at 50-100 V until the dye migrated ¾ th distance of the agarose gel.
2. Gel was transferred to a tray containing about 200-300 ml of depurination solution and kept in it with gentle shaking for 8-10 minute till the dye colour changed to yellow.
3. The solution was discarded and the gel was washed with sterile glass distilled water.
4. The gel was transferred to a tray containing 250 ml of denaturation solution and was allowed to gently shake for half an hour till the dye colour changed to blue. The solution was decanted and rinsed with sterile glass distilled water.

5. The DNA was then transferred from the gel to Hybond N+ membrane (Amersham, UK) by capillary transfer overnight using 0.5 N NaOH - 0.5 N NaCl solution.
6. The next day, the membrane was removed from the assembly and then washed briefly with 2 X SCC. The membrane was baked at 80^{o}C for 3 hours to fix the transferred DNA fragments.

Random primer labeling

Random primer labeling of the DNA probes (Feinbeng and Vogelstein, 1983) was done using the Megaprime DNA labeling kit (Amersham, UK). Reaction (50 µL) was set up as follows:

Table 5.2: Components of random primer labeling

Components	Volume (µl)
25 ng DNA (used as probe)	5.0
Primer solution (random hexamers(3.5A 260 U))	5.0
Final volume	10.0

The mix of DNA and water was denatured in a boiling water bath for 10 min and chilled in ice. Immediately the primer was added and this reaction mixture was added to the following reaction mixture.

Table 5.3: Components of the reaction mixture

Components	Volume
10 X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM MgCl2; 10 mM DTT; 0.5 mg/mL acetylated BSA)	5.0 µL
dATP (0.5 mM)	4.0 µL

dGTP (0.5 mM)	4.0 µL
dTTP (0.5 mM)	4.0 µL
(α-32P)-dCTP (Sp. activity 3000 Ci/mmol)	5.0 µL
Sterile deionized water	16.0 µL
Exonuclease free Klenow fragment (2 U/ µL)	2.0 µL
Total volume	50.0 µL

The labelling reaction was carried out at 37 ºC for 1 h and stopped by heating at 95 ºC in a dry bath for 10 min and snap chilled on ice. This was immediately added to the blots in pre-hybridization mix.

Pre-hybridization and hybridization Solutions

20X SSC: 3 M NaCl; 0.3 M Sodium citrate (pH 7.0)

Hybridization buffer: 1% BSA; 1.0 mM EDTA, pH 8.0; 0.5 M Sodium phosphate, pH 8.0; 7% SDS

Low stringency wash buffer: 2 X SSC, 0.1% SDS

High stringency wash buffer: 0.2 X SSC, 1% SDS

The blots made as in section 2.8.4 mentioned were pre-hybridized at 62ºC in 30 ml of hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted and fresh buffer added with the denatured radiolabelled probe. Hybridization was carried out at 60ºC for 14-18 h. The solution was discarded and the membrane washed with low stringency buffer at 55-65 ºC for 5 min followed by a high stringency wash at 55-65 ºC for 5 min. The moist blot was wrapped in saran wrap and placed in the developing / intensifying screen provided with the Typhoon TRIO+ scanner. After 6-8 h of exposure, remove the membrane and scan the screen with Typhoon trio scanner system. One can increase the exposure time if the band intensity is weak. The membrane scanning is done usually in the resolution mode instead of sensitivity mode of the scanner. Good quality of band pictures can be obtained by scanning at 500 micron resolution with the system. This instrument will provide many more options and functions to get exquisite band pictures of the exposed membrane. After scanning the intensifying screen, the signals can easily be erased from it, upon exposure to a white light provided with the system for 10 min and the same screen can be used to develop the other membrane.

Sequence analysis

The cloned gene sequence was translated into the protein sequence using the `sequence translation tool' at the ExPasy server (http://www.expasy.ch/tools/). Sequence analyses was performed with the BLAST program of the National Centre for Biotechnology Information (NCBI), NIH, Bethesda,MD, USA. Sequence alignments were obtained using CLUSTALW. The α-amylase of the *Streptomyces lonarensis* strain NCL 716 was designated Amy Strplo 716 with the GenBank accession number ADZ45287, and the numbering starts with the first amino-acid of the partial sequence. The UPGMA tree was developed using the tree construction option of CLUSTALW multiple sequence alignment program. Codon usage was computed using the CAIcal server (http://genomes.urv.es/CAIcal).

Results and Discussion

Standardization of genomic DNA isolation from Streptomyces lonarensis strain NCL 716

Genomic DNA was initially isolated using Kirby's protocol (1967). However, the isolated DNA could not be digested with restriction enzymes nor could it be amplified by PCR. Hence DNA was isolated by using five additional methods as described in 'Materials and Methods'. The quality and concentration of the isolated DNA was determined by loading an aliquot on a 0.8% agarose gel and comparing its concentration with a known quantity of lambda DNA. The quality of DNA was also checked by digesting it with a few restriction enzymes and religating the digests (Fig. 5.1, 5.2, 5.3, 5.4, 5.5).

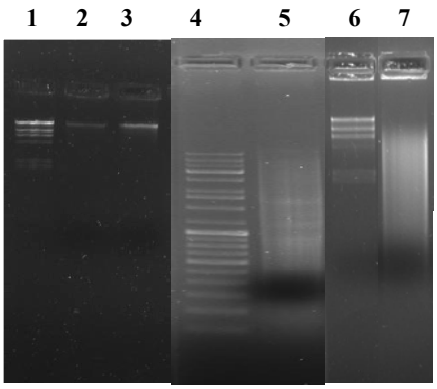

Fig 5.1

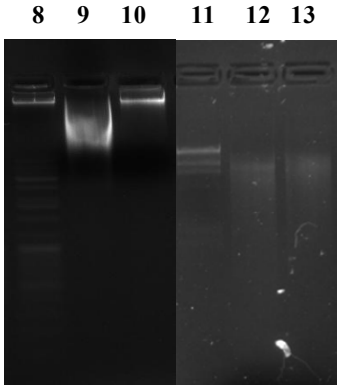

Fig 5.2

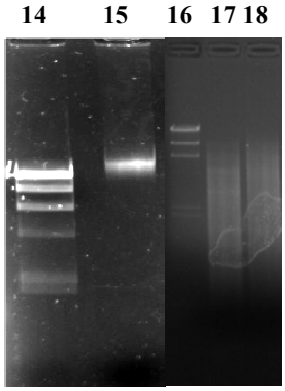

Fig 5.3

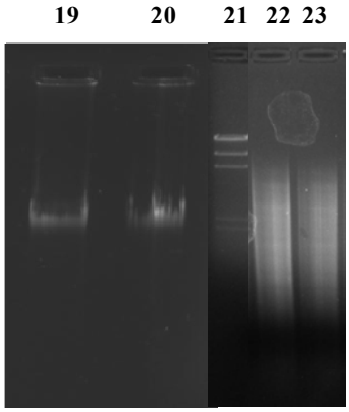

Fig 5.4

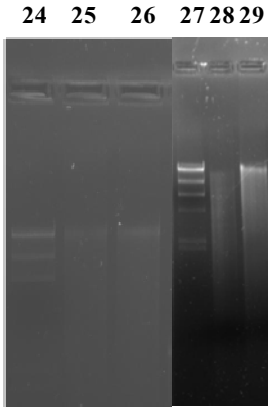

Fig 5.5

Fig 5.1: DNA isolated by PrepEase® Genomic DNA isolation kit from USB Corporation, USA**; Fig 5.2 :** DNA isolated by CTAB method (Dellaporta *et al*, 1983)**; Fig. 5.3:** DNA isolated by the protocol of Crowley *et al*, (2003)**; Fig. 5.4:** DNA isolated by the protocol of Kieser *et al,* (2000)**; Fig. 5.5:** DNA isolated by the protocol of Wang *et.al,* (2005)**.**

Lane 1,5, 11,14,16,21, 24, 27: λ-*Hind III* marker**; Lane 4,8:** Medium range marker**; Lanes 2,3,9,10,15,19,20,25,26**: Total genomic DNA**; Lanes 5,11, 17,22,28**: Digestion of DNA with BssH II**; Lanes 12, 18, 23, 29**: Ligation of the digest.

The yield of DNA obtained by each of the above mentioned methods is shown in Table 5.4.

Table 5.4 DNA yield obtained by various DNA isolation protocols

Method No.	Reference	Yield of DNA per g of cell mass
1	USB Genomic DNA Extraction kit	20 µg
2	CTAB method (Dellaporta, 1983)	5 µg
3	Crowley (2003)	80 µg
4	Kieser *et al* (2000)	150 µg
5	Wang et.al. (2005)	75 µg

Though the yield of DNA isolated using Methods 3 and 4 was relatively high as compared with methods 1 and 2, the restricted DNA did not ligate. Polysaccharides and other impurities are known to inhibit ligation. The DNA isolated by Methods 1, 2 and 5 could easily be digested by different restriction enzymes and the restricted DNA could also be ligated. Since the yield of DNA /g of cell mass obtained by Method 5 were high, this method was used in subsequent experiments.

Streptomyces produce an extensive branching substrate and aerial mycelium. The substrate hyphae are approximately 0.5-1.0 µm in diameter and often lack cross-walls during the vegetative phase. Growth occurs at the hyphal apices and is accompanied by branching, thus producing a complex tightly woven matrix of hyphae during the vegetative growth phase (Anderson and Wellington, 2001). Isolation of *Streptomyces* DNA has been hindered by the general resistance of different strains to lysis. DNA isolation often requires lengthy extraction and purification procedures (Hopwood *et al*, 1985). The most common problems encountered while isolating DNA from *Streptomyces* species with published methods are (a) poor yields of DNA and (b) high polysaccharide contaminants. Low DNA yields may be due to incomplete lysis of the *Streptomyces* cells. The tendency of the organism to grow as compact masses or pellets of mycelium may be the reason for this problem. (Tripathi and Rawal, 1998)

Large amount of acidic polysaccharides are one of the limiting factors in DNA extraction, i.e., sulfated polysaccharides (e.g., agar, carrageenan and fucan) and carboxylic polysaccharides (e.g., alginic acid) (Su and Gibor, 1988). These acidic polysaccharides are high density polysaccharides and are more water soluble than the neutral polysaccharides of land plants and their solutions are highly viscous (Mackie

and Preston, 1974). It is well known that polysaccharides interfere with the DNA extraction process by absorbing DNA. The polysaccharides co-precipitate with DNA during the extraction procedures leading to problems, such as poor yields and subsequent interference with restriction endonuclease digestion (Chan and Goodwin, 1995; Nair *et al*, 1999 ; Wang *et al*. 2005).

Several workers have used different protocols and modifications for isolating good quality DNA from *Streptomyces* (Kirby *et al*, 1967; Chater *et al*, 1989; Tripathi and Rawal, 1998; Kieser *et al*, 2000). The protocol by Kirby *et al* (1967) uses a mixture of phenol and cresol to efficiently remove proteins. This method which has been commonly used for isolation of *Streptomyces* DNA has the advantage that polysaccharides do not precipitate along with the DNA. Tripathi and Rawal (1998) used lysozyme to dissolve the cell wall and the resulting protoplasts were lysed with detergent. Liquid nitrogen was used to break the mycelium pellets thus increasing the surface area so that lysozyme could efficiently dissolve the cell wall. The polysaccharides in the DNA preparation were removed with cetyltrimethylammonium bromide.

In the present work, isolation of high yield of good quality DNA was accomplished by eliminating the contaminating polysaccharides in the very first step of DNA isolation by washing the cell mass with polysaccharide eliminating buffer as described by Wang *et.al,* (2005). Though Wang *et al* (2005) used this protocol to eliminate acidic polysaccharides from the brown alga *Laminaria japonica,* this method has proved efficient in the isolation of good quality DNA from *Streptomyces lonarensis* strain NCL 716.

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Studies on the methylation status of the DNA using methylation sensitive restriction enzyme isochizomers.

The restriction enzymes commonly used to assess the status of cytosine and adenine methylation in DNA are:

(i) Isoschizomers *Msp I* and *Hpa II*

(ii) Isoschizomers *Mbo I and Sau 3AI*

Msp I and *Hpa II* are isoschizomers and they recognize the sequence 5‘CCGG3’. They are insensitive to dam methylation and sensitive to dcm methylation. *MspI* and *Hpa II* cannot cut the sequence 5’CCGG3’ when the external C is methylated. Hpa II is sensitive to CpG methylation i.e it will not cut if internal C in $^{5'}$CCGG$^{3'}$ is methylated,

while *Msp I* is insensitive to it. As *Msp I* and *Hpa II* digest the DNA extensively to the same extent, it appears that the CCGG sequences in the genome are not methylated (Fig 5.6). Moreover, the DNA is extensively digested by these two enzymes suggesting that the DNA is GC rich. This is a characteristic of *Streptomyces* DNA (Madigan, 2005).

Mbo I and *Sau3AI* are isoschizomers and they recognize the sequence $^{5'}$GATC$^{3'}$. *Mbo I* will cleave dam sites which lack adenomethylation and its activity is blocked by complete dam methylation and probably by hemi-methylation as well. *Sau3AI* will cleave all dam sites regardless of adenomethylation but is completely blocked by methylated cytosines within the dam sequence. The *Streptomyces lonarensis* strain NCL 716 DNA is digested to the same extent by *Mbo I* and *Sau 3AI*, suggesting that the GATC sequences are not methylated in the A or C.

Methylation of cytocines and adenine have been shown to play a role in gene regulation (David *et al*, 2001).

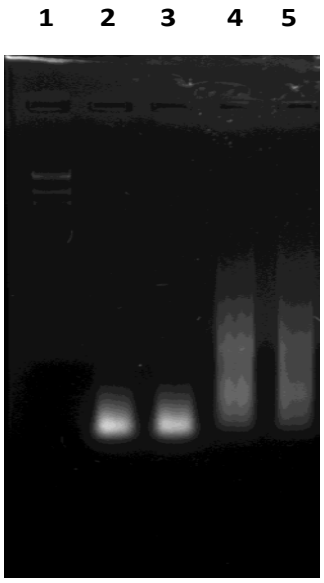


Fig. 5.6: Digestion of *Streptomyces lonarensis* strain 716 DNA with methylation sensitive restriction enzymes.

Lane 1: λ-*Hind III* molecular weight marker; **Lane 2:** *Msp I*; **Lane 3:** *Hpa II*; **Lane 4:** *Mbo I*;**Lane 5:** *Sau3A I*

Cloning of the gene encoding the alkaline α-amylase from the Streptomyces lonarensis strain NCL 716.

The sequence of the partial amylase gene encoding 1306 bp was cloned using a combination of strategies as described below:

(i) Stretches of the gene that was flanked by conserved regions were cloned by a PCR based approach, by using primers designed as per the conserved regions found in *Streptomyces* α-amylase genes.

(ii) The sequence of the regions 5' upstream and 3' downstream to the above stretch did not have conserved regions and hence was cloned by using the Universal Genome Walker Kit (Clontech Takara) and a *Sma I* genomic library.

(i) Cloning of the gene by the PCR based approach, using primers designed as per the conserved regions found in Streptomyces α-amylase genes.

```
P09794      ---MARRLATASLAVLAAAATALTAPTPAAAAPPGAKDVTAVLFEWKFAS
P30270      ---MARRLATASLAVLAAAATALTAPTPAAAAPPGAKDVTAVLFEWKFAS
P22998      ---MARKTVAAALALVAGAAVAVTGNAPAQAVPPGEKDVTAVMFEWNFAS
O65947      -------MARRTLAGAARSRALVMTPTTAQASPPGTKDVTAVLFEWKYDS
P27350      --MASRTLSGALALAAAATAVLAAPATVAHRSPPGTKDVTAVLFEWDYVS
P08486      MQQRSRVLGGTLAGIVAAA-AATVAPWPSQATPPGQKTVTATLFERKYVD
Q59964      MQHRFRLIGGTLAGVLTVAGLTTLAPWQSQATPPGEKTVTVTMFERPYAD
                          : :         :   *** * **..:**  : .
P09794      VARACTDSLGPAGYGYVQVSPPQEHIQGSQWWTSYQPVSYKIAGRLGDRA
P30270      VARACTDSLGPAGYGYVQVSPPQEHIQGSQWWTSYQPVSYKIAGRLGDRA
P22998      VARECTDRLGPAGYGYVQVSPPQEHLQGGQWWTSYQPVSYKIAGRLGDRT
O65947      VAKECTSHLGPG-LPYVQVSPPAEHIQGSQWWTSYQPVSYKIAGPVGDRT
P27350      VAKECTSTLGPAGYGYVQVSPPAEHIQGSQWWTSYQPVSYKIAGRLGDRA
P08486      VAKACTDQLGPAGYGYVEVSPASEHIQGGQWWTSYQPVSYKIAGRLGDRD
Q59964      VASACTDQLGPAGYGYVQVSPATEHIQGDQWWTSYQPVSYRIAGRLGDRD
            **  **. ***.   **:***. **:**.**********:*** :***
                                  ---------->
P09794       AFKSMVDTCHAAGVKVVADSVINHMAAGSGTGTGGSAYQKYDYPGIWSGA
P30270       AFKSMVDTCHAAGVKVVADSVINHMAAGSGTGTGGSAYQKYDYPGIWSGA
P22998       AFKNMIDTCHAAGVKVVADSVINHMANGSGTGTGGTSFSKYDYPGLYSGS
O65947       AFRNMVNTCHAAGVKVVADTVINHMSAGSGTGTGGSSYTKYSYPGLYSSY
P27350       AFRSMVNTCHAAGVKVVVDTVINHMSAGSGTGTGGSSYTKYDYPGLYSAP
```

```
P08486        AFASMVSACHAAGVKVIADAVVNHMAAGSGR--HHAQYTKYNYPGFYQDQ
Q59964        GFAAMVDACHSAGVKVVADAVINHMAAGSGTGTGGTAYTKYDYPGYFGDA
                 .*  *:.:**:*****:.*:*:***: ***     : : **.*** :
P09794       DMDDCRSEINDYGNRANVQNCELVGLADLDTGESYVRDRIAAYLNDLLSL
P30270       DMDDCRSEINDYGNRANVQNCELVGLADLDTGEPYVRDRIAAYLNDLLLL
P22998       DMDDCRATISNYQDRANVQNCELVQLPDLDTGEDHVRGKIAGYLNDLASL
O65947       DFDDSTSQVTNYQDRWNVQHCELVGLADLDTGESYVRGGIARYMNDLLSL
P27350       DFDDCTAEITDYQDRWNVQHCELVGLADLDTGEEYVRQTIAGYMNDLLSL
P08486       TFHGCRKSISDYTNRDDVQTCELVDLADLGTGSDYVRTTIAGYLG-LRSL
Q59964       DFHTCRTAIKDYTDRGDVQNCELVGLSDLDTGKDEVRSTIAAYLDGLRSM
               :. .   :.:* :* :** **** *.**.**.  **  ** *:. *  :
P09794       GVDGFRIDAAKHMPAADLTAIKAKVGNGSTYWKQEAIHGAGEAVQPSEYL
P30270       GVDGFRIDAAKHMPAADLTAIKAKVGNGSTYWKQEAIHGAGEAVQPSEYL
P22998       GVDGFRIDAAKHMPAADLANIKSRLTNPNVFWKLEAIHGAGEAVSPSEYL
O65947        GVDGFRIDAAKHIDAADLANIKSRLTNPSVYWKQEVIYGAGEAVQPTEYT
P27350       GVDGFRIDAATHIPAEDLANIKSRLSNPNAYWKQEVIYGAGEPPKPGEYT
P08486       GVDGFRIDAAKHISATDLAAVKGKMKDP-GFWVQEVIYGAGEAVRPDEYT
Q59964        GVDGFRIDAAKHMAADDVAIQGQDERPP-GSGSPEVIQGGGEAVQPEEYT
              ********** *: * *::              *.* *.**.  * **
P09794      GTGDVQEFRYARDLKRVFQNENLAHLKNFGEDWGYMASGKSAVFVDNHDT
P30270       GTGDVQEFRYARDLKRVFQNENLAHLKNFGEDWGYMASGKSAVFVDNHDT
P22998       GSGDVQEFRYARDLKRVLQGEKLSYLKNFGEAWGHMPSGQSGVFVDNHDT
O65947       GNGDVQEFPYAYDLKRVFNNENLAYLKNYGEAWGYLNSSVAGVFVDNHDT
P27350       GTGDVQEFRYAYDLKRVFTQEHLAYLKNYGEDWGYLSSTTAGVFVDNHDT
P08486        GIGDVDEFRYGTHLKSAFQSGNIAQLKSVADGK--LWQRQARTFVDNWDT
Q59964        EIGDVDEFRYGGHLKSAFQGGGIAQLKAVADRK--LGSASARTFVDNWDT
                 ***:** *. .** .:    :: **  .:    : .  : .**** **


P09794       ERGGDTLNYKNGSAYTLAGVFMLAWPYGSPDVHSGYEFTDHDAGPPNGGT
P30270       ERGGDTLNYKNGSAYTLAGVFMLAWPYGSPDVHSGYEFTDHDAGPPNGGT
P22998       ERGGDTLSYKDGANYTLASVFMLAWPYGSPDVHSGYEWTDKDAGPPNNGQ
O65947       DANGSTLNYRDGANYTLANVFMLAWPYGAPDINSGYEWSDAAAGPPN-AT
```

```
P27350      ERNGSTLNYKNDATYTLANVFMLAWPYGAPDINSGYEWSDPDARPPDGGH
P08486       ERNGSTLTYKDGAAYTLANVFMLASPYGSPNVYSGYEWTDKDAAAG----
Q59964      ERNGSTLTYKDGAAYTLANVFMLASPYGSPNVYSGYEWSDADAGPPS---
             : .*.**.*::.: ****.***** ***:*:: ****::*  * .
P09794      VNACYSDGWKCQHAWPELSSMVGLRNTASGQPVTNWWDNGGDQIAFGRGD
P30270      VNACYSDGWKCQHAWPELSSMVGLRNTASGQPVTNWWDNGGDQIAFGRGD
P22998      VNACYTDGWKCQHAWREISSMVAFRNTARGQAVTNWWDNGNNAIAFGRGS
O65947      VNACWQDGWKCQHKWPEIIRMVGFRNATRGQAVTDWWDNGGDAIAFGRGS
P27350      VDACWQNGWKCQHKWPEIASMVAFRNATRGEPVTDWWDDGADAIAFGRGS
P08486      ----GSTGWTDDAAKREITGMVGFRNAVGSAELTNWWDNGGRPLAFARSD
Q59964      ----GADGWTDTHAQQTITGLVGFRNAVGSAELTDWWDNGGSALAFGRGD
                   **.       :  :*.:**:. . :*:***:*   :**.*..
P09794      KAYVAINHEGSALNRTFQSGLPGGAYCDVQSGRSVTVGSDGTFTATVAAG
P30270      KAYVAINHEGSALNRTFQSGLPGGAYCDVQSGRSVTVGSDGTFTATVAAG
P22998      KAYVAINHETSALTRTFQTSLPAGSYCDVQSNTPVTVNSSGQFTATLAAN
O65947      KGFVAINHESATISRTYQTSLPPGPYCNVQNNTTCSVNGSGQLTATLAAN
P27350      KGFVAINHESATVQRTYQTSLPAGTYCDVQSNTTVTVDSAGRFTAALGPD
P08486      KGFVALNNGDAALTQTFATSLPAGTYCDVVHAASS-CDGDTVTVGDTEAQ
Q59964      KGFVALNNADDALTETFTTSLPAGTYCNVAAASPDDCDGNTVTVGDDGAV
             *.:**:*:   :: .*: :.** *.**:*    .   ..    ..   .
P09794      TALALHTGARTCSGGGTGPGTG---QTSASFHVNATTAWGENIYVTGDQA
P30270      TALALHTGARTCSGGGTGPGTG---QTSASFHVNATTAWGENIYVTGDQA
P22998      TAVALHVNATGCGSTPTTPPTTPPATSGASFNVTATTVVGQNIYVTGNRA
O65947      TALAVYAGKSSC-------------------------------------
P27350      TALALHNGRTSC-------------------------------------
P08486      VDAAKSVALHVGATGQSACRQAVALHVPGQSAGSPRSSAKRVEQ------
Q59964      QATVPARGALALHTGAQAG-------------------------------
                    .
P09794      ALGNWDPARALKLDPAAYPVWKLDVPLAAGTPFQYKYLRKDAAGKAVWES
P30270      ALGNWDPARALKLDPAAYPVWKLDVPLAAGTPFQYKYLRKDAAGKAVWES
P22998      ELGNWAPASALKLDPATYPVWKLTVGLPAGTSFEYKYIRKDAAGNVTWES
```

```
O65947      ------------------------------------------------
P27350.     ------------------------------------------------
P08486      ------------------------------------------------
Q59964      ------------------------------------------------

P09794      GANRTATVGTTGALTLNDTWRG
P30270      GANRTATVGTTGALTLNDTWRG
P22998      GANRTATVPASGQLVLNDTFRS
O65947      ----------------------
P27350      ----------------------
P08486      ----------------------
Q59964      ----------------------
```

Fig 5.7 Clustal protein sequence alignment of a few *Streptomyces* α-amylases. The conserved regions on the basis of which primers were designed are underlined and the primers are indicated by arrows.

Following criteria were taken into consideration while designing the primers:

1. Primers should be 15-28 bases in length. Base composition should not exceed 50% (G+C).
2. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming.
3. The difference in the Tm between the two primers should not exceed 5°C.
4. 3'ends of primers should not be complementary, otherwise primer dimers will be synthesized preferentially to any other product.
5. Primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided.
6. Runs of three or more C's or G's at the 3' ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing) and should be avoided.

Table 5.5: Details of primers designed for cloning the α-amylase gene.

Primer	Sequence	Tm	Length (bp)	GC %	
SF2	5’ TTCCGGATCGACGCCGCCAAGCA 3’	82.7	23	62.2	Gene forward
S9	5’CGACTCGGTCATCAACCACAT 3’	67.2	21	52.8	Gene forward
S7	5’ TGTCGTGGTTGTCGACGAAG 3’	67.4	20	55	Gene reverse
SAR3	5’GGGTAGTCGTACTTGGTGTAC 3’	59.8	21	59.8	Gene reverse
SAR4	5’ CATGTGGTTGATGACCGAGTC 3’	59.8	21	52.4	Gene reverse
SAF1	5’GTACCTGGACGACTTCGGCCA 3’	58	21	61.8	Gene forward
SAF2	5’CGCCTTCGACATCAAGCGCATC 3’	59	22	99	Gene forward
STF1	5’ AAGGCGAGAAGGACGTCA 3’	58.6	19	57.9	Gene forward

Standardization of amplification conditions

Initially when genomic DNA was used as a template for PCR using the designed primers, no amplification product was visible on the agarose gel. Hence, different additives such as DMSO, formamide, glycogen, BSA, Tween 20 and Triton X-100 were added. (Fig. 5.8 and Table 5.6) . Among the additives added, the band of expected size (~540 bp) with primers S9-S7 amplified when DMSO, formamide, BSA and Triton X-100 was added to the PCR mix. Since addition of DMSO, resulted in a bright amplicon, DMSO was used as the additive for all further PCR amplifications.

Additive	Volume used in 25 µl of PCR reaction	Final Conc (%)
Formamide	2 µl	8
DMSO	1.25µl	5
Glycogen	2.5 µl	10
BSA	2.5µl	10
Tween 20	0.5 µl	0.2
Triton X 100	0.5 µl	0.2

Table 5.6 : Different additives used for amplification of genomic DNA

The PCR program was as follows:

Cycle 1: 94° C – 5 minutes (initial denaturation)

Cycle 2: 94° C – 30 seconds (denaturation)

68° C – 45 seconds (Annealing)

72° C – 2 minutes (Extension)

Cycle 3: 72° C – 5 minutes (Final Extension)

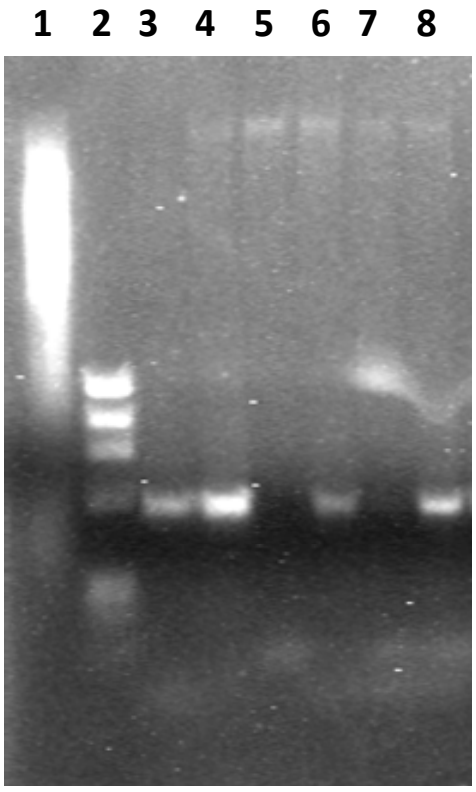


Fig: 5.8: Amplification of genomic DNA using different additives

Lane 1: λ-Hind III molecular weight marker; **Lane 2:** ΦX 174-*Hae III* Digest; **Lane 3**: formamide; **Lane 4:** DMSO; **Lane 5:** glycogen; **Lane 6:** BSA; **Lane 7:** Tween 20; **Lane 8**: TritonX-100.

Amplification of genomic DNA using pairs of conserved primers

Stretches of the α-amylase gene were amplified using the following pairs of primers:

(i) SF2-S7 (**Fig 5.9 A**) **(ii)** S9-S7 **(Fig 5.9 B) (iii)** STF1-SAR3 **(Fig 5.9 C) (iv)** SAF2-STR2 (**Fig 5.9 D, lane j**) **(v)** STF1-STR2 (**Fig 5.9 C, lane f**) **(vi)** S9-STR2 (**Fig 5.9 D, lane h**)

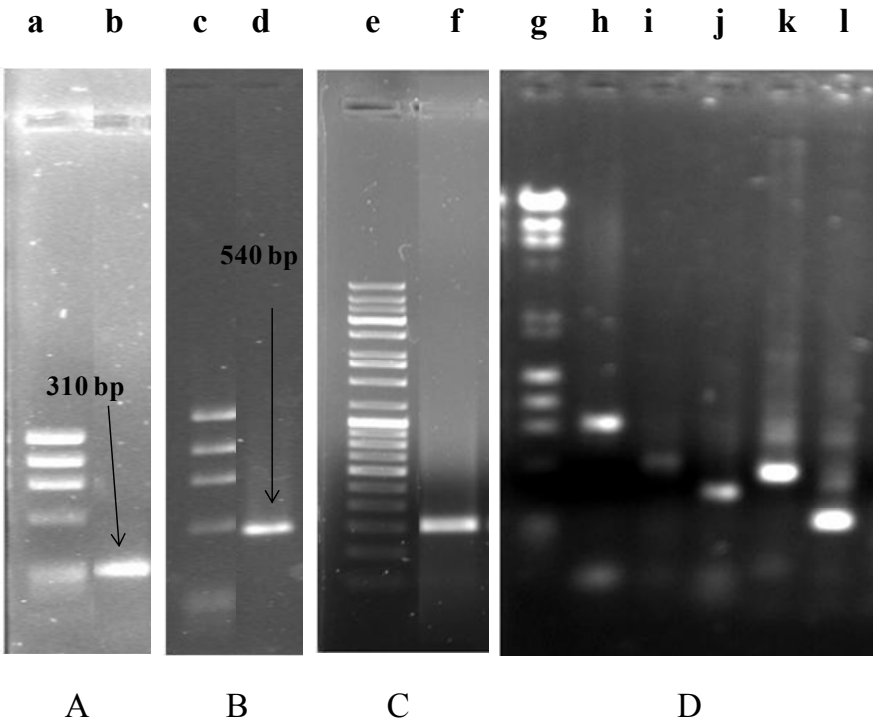


Fig 5.9: Amplification of genomic DNA using pairs of conserved regions

Lane a,c,g: φX174λ-*Hind III* molecular weight marker; **Lane b:** SF2-S7; **Lane d:** S9-S7; **Lane e:** medium range marker; **Lane f:**STF1-SAR3; **Lane h:** S9-STR2; **Lane i:** Nested PCR of primary PCR product **(h)** with SF2-STR2; **Lane j:** Nested PCR of primary PCR product **(h)** with SAF2-STR2; **Lane k:** Nested PCR of primary PCR product **(h)** with S9-S7; **Lane l:** Nested PCR of primary PCR product **(h)** with SF2-S7.

The PCR products were confirmed to be a part of the α-amylase gene by performing a nested PCR on the primary amplification product (**Fig 5.9 D**).

Each amplicon was eluted from the agarose gel and cloned into pCR4 TOPO vector as described in 'Materials and Methods'. Positive clones were sequenced and the stretch of the gene flanked by primers STF1 and STR2 was derived by overlapping the sequence of the clones of the above amplicons. A stretch of 1137 bases of the α-amylase gene was deciphered using this strategy

(ii) ***Cloning of the regions 5' upstream and 3' downstream to the above deciphered stretch using the Universal Genome Walker Kit (ClonTech Takara) and a Sma I genomic library.***

Principle of the Genome Walker Universal Kit (ClonTech Takara)

Genome Walker DNA walking is a simple method for cloning unknown genomic DNA sequences adjacent to a known sequence. In this strategy, the first step is to digest the genomic DNA with a restriction enzyme which cleaves the DNA to generate blunt ends. Each fragment of digested genomic DNA is then ligated to the Genome Walker Adaptor. Such pools of uncloned, adaptor-ligated genomic DNA fragments are referred to as Genome Walker libraries. The library is used as a template and a primary PCR is carried out using an outer adaptor primer (AP1) provided in the kit and an outer gene-specific primer (GSP1). The primary PCR product is then diluted and used as a template for a secondary or nested PCR which uses a nested adaptor primer (AP2) and a nested gene specific primer (GSP2). Each of the DNA fragments which begin in a known sequence at the 5'end of GSP2 and extend into the unknown adjacent genomic DNA can then be cloned and further analyzed. (Fig 5.10)

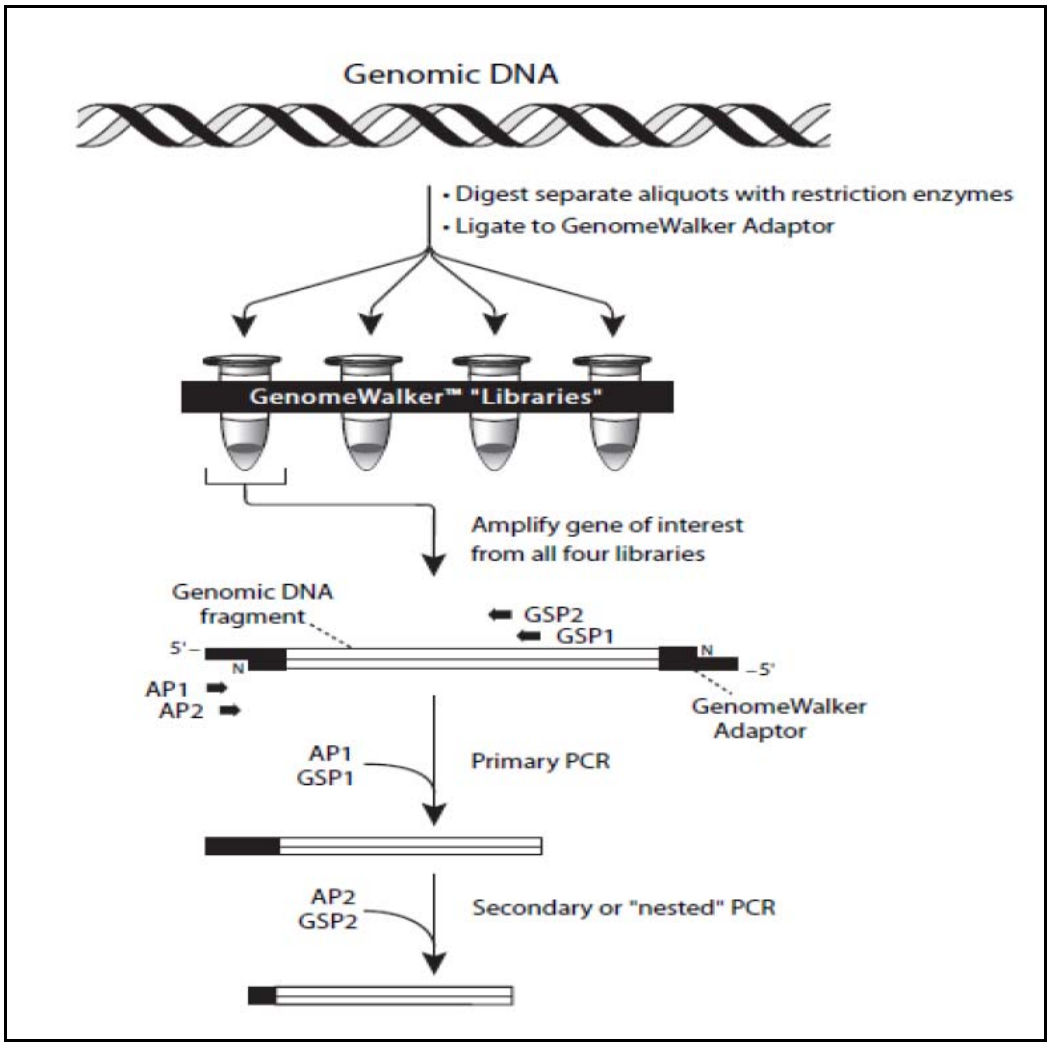


Fig 5.10 Genome Walking Method by using Genome Walker™ Kit

(Ref : Genome Walker Universal Kit Manual)

The genomic DNA of the *Streptomyces lonarensis* strain NCL 716 was digested with enzymes such as *Sma I, Stu I, Eco RI, Eco RV and Dra I* which create blunt end restriction fragments. Of these, only *Sma I* gave a good digest of the genomic DNA and hence was used for this strategy (Fig 5.11 a). A Southern blot having the *Sma I* digest along with the amplicons derived using primers SF2-S7 (~ 280 bp) and S9-S7 (~ 540 bp) was made (Fig 5.11a). The blot was hybridised with the

SH2-S7 derived probe of ~280 bp probe as described in Materials and Methods. The autoradiogram is shown in Fig 5.11 b. *Sma I* has only two sites within the derived sequence (Fig 5.12). In the autoradiogram, the probe hybridised to two fragments of sizes ~ 250 bp and 800 bp. The ~ 250 bp fragment corresponds to the central fragment

in the derived sequence delimited with *Sma I* sites. The second fragment of ~800 bp corresponds to the *Sma I* fragment of the gene towards the 3' end of the gene i.e extending 3' downstream from the 246 bp *Sma I* fragment. From the derived sequence, the stretches SF2-STR2 and the second *Sma* I site –STR2 are 606 bp and 554 bp respectively. Hence, the expected size between the second *Sma I* site to the *Sma I* site in the unknown 3'region should be ~ 732 bp. This size of fragment is seen on the autoradiogram. Hence, the *Sma I* digest was purified and ligated to the genome walker adaptors according to the manufacturer's instructions. PCR was performed using gene specific primers and adaptor specific primers AP1 and AP2. For amplifying the upstream region, GSPs used in combination with AP1 and AP2 were SAR3 and SAR4. For amplifying the 3' downstream region, the GSP used in combination with AP1 and AP2 was SAF2 (Figs.5.18 and 5.19). The PCR fragments were excised from the gel using SNAP columns and cloned into pCR4 TOPO and recombinants were selected as described in Materials and Methods. By sequencing of the plasmids, the gene could be extended by 150 bp downstream to STR2. One *Sma I* site was found immediately 5' upstream to STF1. Hence, no additional sequence 5' to STF1 could be derived using this strategy. Hence, using the Genome Walker strategy and the PCR strategy described in the earlier section, 1312 bp of the α-amylase gene could be deciphered

Fig: 5.12(A): Partial gene sequence of *Streptomyces lonarensis strain NCL 716 (Amy Strplo 716).* *Sma I* sites within the sequences are highlighted in yellow. Primer sequences are indicated in colour and shown by arrows.

STF1

5'**GGCGAGAAGGACGTCAC**CGCCGTCCTCTTCTCCTGGGACTTCGACTC

GATCGCCCGCGAGTGCACCGACCGGCTCGGCCCGGCCGGCTACGGCTTC

GTGCAGGTCTCCCCGCCGCAGGAGCACATCCGGCTCCCAGTGGTGGACC

CAGTACCAGCCCGTCAGCTACGACATCGCCGGACGCCTCGGCAACGCG

CAGCAGTTCCGGGCGATGGTGAACACCTGCTCCGACGCCGGGGTCGGA

GTGGTGGT**CGACCGTC**A**TCAACCACATG**GCCGCCGGGTCCGGCACCG

S9 SAR4

SAR3

GCACCGGGGGCACCGC**GTACACCAAGTACGACTA**CCCCGGGCTGTAC

GGCAGCCAGGACTTCAACGACTGCCGGCAGGACATCGCCCCCGGGATA

CGCCGGCGACCGGTGGCGGGTCCAGAACTGCGAACTCGTGGGCCTGTC

CGACCTGGACACCGGCAGCGGCTACGTGCAGCAGACCATCGCCGACTA

SF2

CATGAACACCCTGCTCGACTGGGGCGCGCGGC**TTCCGGGTCGACGCG**

GCCAAGCACATCCCGGCCCAGCACCTGGAGCAGATCCGCGCCCGGGTC

GGCGACGGATCGGTCTACTGGAAGCAGGAGATGATCTACGGGGCGGGC

GAGGCGATCACCCGACGAGTACACGGGCGCGGGCGACGTGCAGGAGTT

SAF2

CCGCCA**CGCCTTCGACATCAAGCGCATC**TTCCAGAACGAACGCCTCG

CGTACCTGGACGACTTCGGCCAGAGCTGGGGCTACCTGCCTCCGCGGCG

S7

CCGGCGT**CTTCGTCGACAACCACGACA**CCGAGCGCAACGGCTCCACC

CTCACCTACCGCGACGGCTCCGCCTACACCCTCGCCAACGTCTTCATGC

TGGCGTGGAACTACGGCAGCCCCGACATCCACTCGGGGTACGAGTTCAC

CGACTACGACGCCGGCCCGCCGCGCGGCGGCGGTGCGCGACTGCTACC

AGGACAACTGGAAGTGCCAGCACGCCTGGCCCGAGATCGCGTCCATGG

TCGGGTTCCGCAACGCGGTCGGCGACGCCGCCGTCACCAACTGGTGGG

ACAACGGCAACGACTATCGCCTTCGGCCGCGGCGACCGCGGCTTC**GTC**

STR2

GTCGTCAACCACGAGTCCGCCGCCGTCGACCGCACCTGGCAGAGCTCG

CTGCCCGGCGGAACCTACTGCGACGTGCAGTCCGGACGCGCCGTACGTC

GACGGCTCCGGCCGGTTCACCGCCTCGGTCGCCGCCGGCACCGCCCTGG

CGCTCCACGCCGGCGCCCGCGACTGCTCCGGCAACGGCGGCCC 3’

Fig: 5.12 (B) Translated protein sequence of Amy Strplo 716. The conserved regions are highlighted in yellow colour. The three active site residues D and E are highlighted in blue.

GEKDVTAVLFSWDFDSIARECTDRLGPAGYGFVQVSPPQEHIQGSQWWTQYQ
PVSYDIAGRLGNAQQFRAMVNTCSDAGVGVVVDSVINHMAAGSGTGTGGTA
YTKYDYPGLYGSQDFNDCRQDIAPGDYAGDRWRVQNCELVGLSDLDTGSGY
VQQTIADYMNTLLDWGVAGFRVDAAKHIPAQHLEQIRARVGDGSVYWKQE
MIYGAGEAIHPDEYTGAGDVQEFRHAFDIKRIFQNERLAYLDDFGQSWGYLPS
AGAGVFVDNHDTERNGSTLTYRDGSAYTLANVFMLAWNYGSPDIHSGYEFT
DYDAGPPRGGEVRDCYQDNWKCQHAWPEIASMVGFRNAVGDAAVTNWWD
NGNDVIAFGRGDRGFVVVNHESAAVDRTWQSSLPGGTYCDVQSGRAVTVDG
SGRFTASVAAGTALALHAGARDCSGNGGP

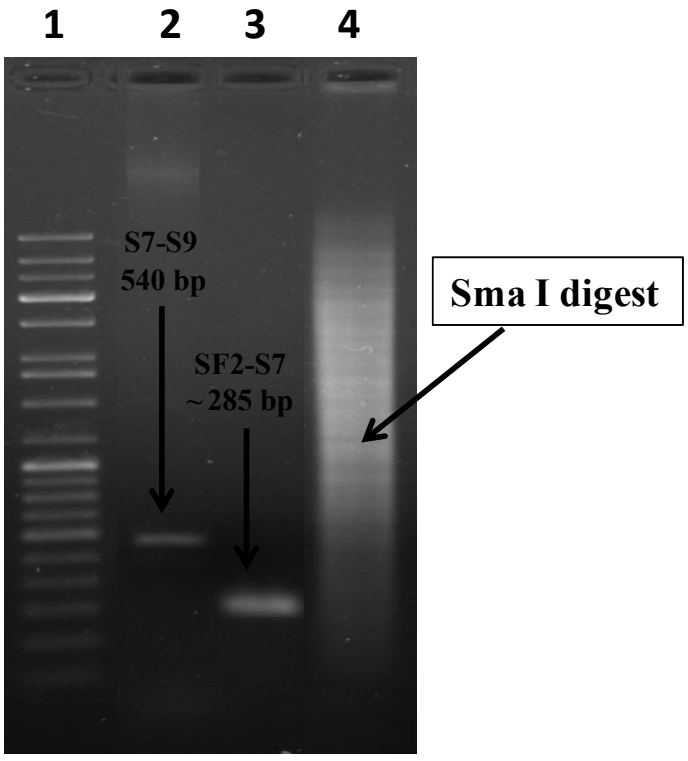


Fig. 5.11 a: Lane 1: Medium range marker; **Lane 2:** 540 bp probe; **Lane 3:** ~ 285 bp probe; **Lane 4:** *Sma I* digest.

Southern hybridization was performed using ~ 285 bp probe (amplicon from SF2 – S7).

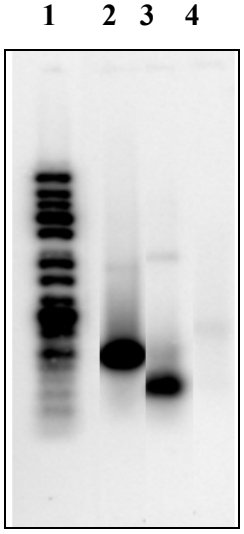
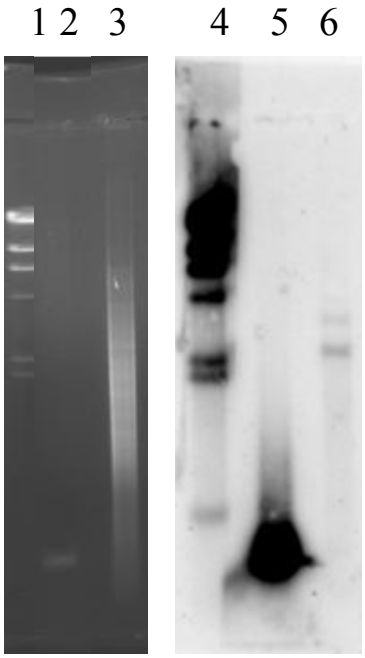


Fig 5.11 b Lane 1: Medium range marker; **Lane 2:** PCR product 540 bp (S7 – S9 amplification); **Lane 3:** ~ 285 bp (probe); **Lane 4:** *Sma I* digest.

In the 1137 bp derived sequence, there were only 2 sites for *Sma I* (Fig 5.12a)

A third strategy based on construction of a library consisting of *BssH II* digested genomic DNA cloned in pNEB 193 was initiated to clone the entire α-amylase gene. There was no site for *BssH II* in the 1312 bp derived α-amylase gene sequence. Hence a Southern blot of genomic DNA digested with *BssH II* was made and was probed with the SF2-S7 probe (~ 285 bp) (Figs 5.13 a & b). The probe hybridised to a band of ~ 2.4 kbp. In addition, it also hybridised to a faint band of ~ 3 kbp. This band could represent a partially digested fragment in the digest carrying the α-amylase gene.



5.13 a **5.13 b**

Fig. 5.13 a : Agarose gel; Fig. 5.13 b : Autoradiogram.

Lanes 1,4: λ-*Hind III* molecular weight marker; **Lanes 2 & 5:** ~ 285 bp probe; **Lanes 3, 6**: *BssH II* digest; **Lane 6:** *BssH II* digest showing two hybridized bands.

The region corresponding to the ~ 2.4 kbp band was eluted from the gel and presence of the gene in the eluted DNA was confirmed using gene specific primers. The eluted DNA was ligated into pNEB193 vector (which was also digested with *BssH II)* and a library was constructed in NovaBlue *E.coli* host. Presently, at the time of submitting this thesis, screening of this library was in progress.

Sequence analysis

The partial sequence of Amy Strplo 716 derived using the strategies described in the earlier sections is 1312 bp long and covers all the conserved regions that are present in all the amylases (Fig 5.12 a and b). It translates into a protein of 437 amino acids, which showed significant homology with the α-amylases from other *Streptomyces.* This sequence has been designated as Amy Strplo 716 (GenBank accession number ADZ45287).

Fig 5.14 shows the ClustalW sequence alignment of Amy Strplo 716 (GenBank Accession number ADZ45287) with the amylases of other *Streptomyces* species which are indicated by their GenBank accession numbers. The conserved regions are in bold and underlined and the three catalytic residues are highlighted.

```
ZP_09178791.1      -------MARRLATASLAVLAAAATALTAPTPAAAAPPGAKDVTAVLFEW
P30270.1|S.griseus ---------------------------------------------------
P09794.1|AMY_STRL  -------MARRLATASLAVLAAAATALTAPTPAAAAPPGAKDVTAVLFEW
ZP_06908805.1      -------MASRTVAAALAVVAGAAVAVTAPSTAQAAPPGDKDVTAVMFEW
YP_001826792.      –MARRSLSAALALVAAAAAALVVPTGFGAAGTARAAAPGDKDVTAVMFEW
ZP_09403579.1      --------------MAGAAALVIPTGFGAAGTAQAAAPGDKDVTAVMFEW
ZP_04707862.1      --MARRPLSAALALVAGAAALVVPTGFGAASPAQAAAPGDKDVTAVMFEW
YP_004925530.      --MARRPLSGALALTMGAAVLAVPAGLGAAGTAQAAPPGEKDVTAVMFEW
YP_004802096.      --MARRSLSASLALVTGAAVLALPAGLGTAGTAQAAPPGEKDVTAVMFEW
NP_631084.1        MHGNTSPARRITATALALTAGVAGSLLATTAPAQASPPGDKDVTAVMFEW
ZP_06526938.1      MHGNTSPARRITATALALTAGVAGSLLATTAPAQASPPGDKDVTAVMFEW
CAB06622.1         MHGNTSPARRITATALALTAGVAGSLLATTAPAQASPPGDKDVTAVMFEW
ABZ81609.1         MHGNTSPARRITATALALTAGVAGSLLATTAPAQASPPGDKDVTAVMFEW
CAA73926.1         MHGNTSPARRITATALALTAGVAGSLLATTAPAQASPPGDKDVTAVMFEW
EHN78434.1         MHGNTSTARRVTATALALTAGVAGSLLATAAPTQASPPGDEDVTAVMFEW
ZP_07314518.1      MHRSTSRARRTTAAALALTAGAAGSLLATAAPAQAAPPGDKDVTAVMFEW
CCA55176.1         ------MARKSVAAALALVAGAAAVAVTGNTAAQATPPGEKDVTAVMFEW
P22998.1|AMY_STRV  ------MARKTVAAALALVAG-AAVAVTGNAPAQAVPPGEKDVTAVMFEW
ZP_06711822.1      ------MARKTVAAALAVLAG-AAAAVTAPTPAQASAPGGKDVTAVMFEW
EHN79425.1         ------MARKSVAAALAVVAG-AAAAVTVPAPAQAAPVGDKDVTAVMFEW
CAL64868.1         ------MANKTVATALALVAG-AAVVVTAPGTAQADPPGGKDVTAVMFEW
ZP_05002170.1      ----MPARAVRAATLSAATALVCATLTAVGPQAVAAPPGEKDVTAVLFEW
ZP_05006689.1      ------MASRPLAAALALVAGAAAAVMAPAGTAQAAPPGAKDVTAVLFEW
AAA96317.1         ------MASRTLSGALALAAAATAVLAAPATVAHASPPGTKDVTAVLFEW
P27350.2|Strepto   ---------------------------------------------------
ADB89947.1|        ------MASRTLSGALALAAAASAVLALPATGAYASPPGTKDVTAVLFEW
ZP_06579441.1      ---------MARRTLAGAVALAAASVVMTPTAAEASPPGTKDVTAVLFEW
ZP_07313341.1      ---MKKEPHMARRTLAVAVALAAASVVMAPTGAQASPPGSKDVTAVLFEW
ZP_07303280.1      ---------MARRFLPAAVALAAAAVVMNPTNAQASPPGAKDVTAVLFEW
ZP_08805833.1      -----MVRRILAWAAALATALTASMIVMPSTEAAASPPGAKDVTAVLFEW
CAA73775.1         -----------MARRTLAGAARSRALVMTPTTAQASPPGTKDVTAVLFEW
AEY88989.1         --------------------------MTPTAAEASPPGTKDVTAVLFEW
BAF49593.1|        --------MARRTLPTAVALTAAALVGMTPTAAEASPPGTKDVTADLFEW
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YP_004816943.      ----MAGNRIPLATALALAAGSVALFALPPQTAQATPPGTKDVTAELFEW
YP_004965987.      ------MASRPLAAALALAAGAAMLIALP-GQAQAAPPGDKDVTAELFEW
ADZ45287           -------------------------------------GEKDVTAVLFSW
ZP_09405139.1      ------MSSRAARATLAGLLAAGGLTVLAPWPSQATPPGEKTVTATLFEW
P08486.1|AMY_STRH  ----MQQRSRVLGGTLAGIVAAAAATVAP-WPSQATPPGQKTVTATLFER
YP_004806014.      ----MQYRFRVIGGTLAGVMTAAGVMTFTPGQSQAAPPGEKTVTVTMFER
AAA82875.1         ----MQHRFRLIGGTLAGVLTVAGLTTLAPWQSQATPPGEKTVTVTMFER


ZP_09178791.1      KFASVARACTDSLGPAGYGYVQVSPPQEHIQGSQWWTSYQPVSYKIAGRL
P30270.1|S.griseu  --------------------------EHIQGSQWWTSYQPVSYKIAGRL
P09794.1|AMY_STRL  KFASVARACTDSLGPAGYGYVQVSPPQEHIQGSQWWTSYQPVSYKIAGRL
ZP_06908805.1      KFDSVARACTDSLGPAGYGFVQVSPPQEHIQGSQWWTSYQPVSYRIAGRL
YP_001826792.      KFASIAKACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
ZP_09403579.1      KFASIAKACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
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YP_004802096.       KFDSVAKACTDSLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYKIAGRL
NP_631084.1         KFTSVAQACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
ZP_06526938.1       KFTSVAQACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
CAB06622.1          KFTSVGQACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
ABZ81609.1          KFTSVAQACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
CAA73926.1          KFTSVAQACTDTLGPAGYGYVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
EHN78434.1          KFTSVAQACTDVLGPAGYGFVQVSPPQEHIQGGQWWTSYQPVSYKIAGRL
ZP_07314518.1       KFDSVAKACTDTLGPAGYGFVQVSPPQEHIQGGQWWTSYQPVSYRIAGRL
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P22998.1|AMY_STRV   NFASVARECTDRLGPAGYGYVQVSPPQEHLQGGQWWTSYQPVSYKIAGRL
ZP_06711822.1       NFASVAKACTDELGPDGYGYVQVSPPNEHIQGSTWWTAYQPVSYKIGTKL
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CAL64868.1          NFASVAKACTDDLGPAGYGYVQVSPPNERVPGPQWWTAYQPVSYRIATKL
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ZP_05006689.1       KFASVARACTERLGPDGYGYVQVSPPQEHIQGPQWWTSYQPVSYKIAGRL
AAA96317.1          DYASVAKECTSTLGPAGYGYVQVSPPAEHIQGSQWWTSYQPVSYKIAGRL
P27350.2|Strepto    -------------------------AEHIQGSQWWTSYQPVSYKIAGRL
ADB89947.1          NYASAAKECTSTLGPAGYGYVQVSPPAEHIQGPQWWTSYQPVSYKIAGRL
ZP_06579441.1       NFASVARECTTTLGPAGYGYVQVSPPAEHIQGAQWWTSYQPVSYKIAGRL
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AEY88989.1          NYASVAKECTTALGPAGYGYVQVSPPAEHIQGAQWWTSYQPVSYKIAGRL
BAF49593.1          NYASVAKECTTALGPAGYGYVQVSPPAEHIQGAQWWTSYQPVSYKIAGRL
NP_827158.1         KFDSVARECTNTLGPAGYGYVQVSPPAEHIQGSQWWTSYQPVSYKIAGRL
ZP_06916602.1       NFASVAKECTTTLGPAGYGYVQVSPPAEHIQGSQWWTSYQPVSYKIAGRL
YP_003492208        NFASVARECTSTLGPNGYGYVQVSPPAEHIQGAQWWTSYQPVSYRIAGRL
YP_004816943        RYDSVAKECTSTLGPAGYGYVEVSPATEHIQGGQWWTSYQPVSYKIAGRL
YP_004965987        TYASVAKECTSTLGPAGYGYVEVSPATEHIQGGQWWTSYQPVSYKIAGRL
ADZ45287            DFDSIARECTDRLGPAGYGFVQVSPPQEHIQGSQWWTQYQPVSYDIAGRL
ZP_09405139.1       KYDAVATACTDTLGPAGYGYVEVSPATEHIQGDQWWTSYQPVSYRIAGRL
P08486.1|AMY_STRH   KYVDVAKACTDQLGPAGYGYVEVSPASEHIQGGQWWTSYQPVSYKIAGRL
YP_004806014        RYADVARACTDELGPAGYGFVQVSPASEHIQGDQWWTSYQPVSYRIAGRL
AAA82875.1          PYADVASACTDQLGPAGYGYVQVSPATEHIQGDQWWTSYQPVSYRIAGRL
                                          *:: *  *** ****** *.  :

ZP_09178791.1       GDRAAFTSMVDTCHAAGVKVVADSVINHMA-----AGSGTGTGGSAYQKY
P30270.1|S.griseu   GDRAAFKSMVDTCHAAGVKVVADSVINHMA-----AGSGTGTGGSAYQKY
P09794.1|AMY_STRL   GDRAAFKSMVDTCHAAGVKVVADSVINHMA-----AGSGTGTGGSAYQKY
ZP_06908805.1       GDRAAFKSMVDTCHAAGVKVIADSVINHMS-----AGSGTGTGGSAYTKY
YP_001826792.       GDRAAFAAMVNTCHAAGVKVIADSVINHMS-----AGSGTGTGGSSYTKY
ZP_09403579.1       GDRAAFSAMVNTCHSAGVKVIADSVINHMS-----AGSGTGTGGSAYTKY
ZP_04707862.1       GDRAAFSAMVNTCHSAGVKVVADSVINHMA-----AGNGTGTGGSAYTKY
YP_004925530.       GDRAAFANMVNTCHSAGVKVIADSVINHMS-----AGSGTGTGGSSYTKY
YP_004802096.       GDRTAFANMVGTCHGAGVKVVADAVVNHMS-----SGSGTGTGGSSYTKY
NP_631084.1         GDRAQFKSMVDTCHAAGVKVVADSVVNHMS-----AGNGTGTGGSSYTKY
ZP_06526938.1       GDRAQFKSMVDTCHAAGVKVVADSVVNHMS-----AGNGTGTGGSSYTKY
CAB06622.1          GDRAQFKSMVDTCHAAGVKVVADSVVNHMS-----AGNGTGTGGSSYTKY
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CCA55176.1          GDRTAFKKMIDTCHAAGVKVVADSVINHMA-----NGSGTGTGGTPFSKY
P22998.1|AMY_STRV   GDRTAFKNMIDTCHAAGVKVVADSVINHMA-----NGSGTGTGGTSFSKY
ZP_06711822.1       GDRAAFKNMVDTCHAAGVKVVADAVINHMA-----NASGTGTAGSSFSKY
EHN79425.1          GDRAAFKSMVETCHAAGVKVVADTVINHMA-----NSSGTGTGGSTYSKY
CAL64868.1          GGRDAFKSMIDTCHAAGVKVVVDTVINHMA-----NSAGTGTGGSSYSKY
ZP_05002170.1       GDRAAFKAMVDTCHAAGVKVVVDSVVNHMAGPADAGATLTGTGGSSYTKY
ZP_05006689.1       GNRADFAAMVNTCHGAGVKVVADAVINHMAAG-----DGVGTGGSPYTKY
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AAA96317.1          GDRAAFRSMVNTCHAAGVKVVVDTVINHMS-----AGSGTGTGGSSYTKY
P27350.2|Strepto    GDRAAFRSMVNTCHAAGVKVVVDTVINHMS-----AGSGTGTGGSSYTKY
ADB89947.1          GDRAAFRSMVDTCHAAGVKVVVDTVINHMS-----AGSGTGTAGSSYTKY
ZP_06579441.1       GDRTAFRNMVNTCHAAGVKVVVDTVINHMS-----AGNGTGTGGSSYTKY
ZP_07313341.1       GDRTAFRNMVNTCHAAGVKVVADTVINHMS-----AGSGTGTGGSSYTKY
ZP_07303280.1       GDRTAFRNMVNTCHAAGVKVVADTVVNHMS-----AGGGTGTGGSSYTKY
ZP_08805833.1       GDRTAFRTMVTTCQAAGVKVVVDTVINHMS-----AGSGTGTGGSSYTKY
CAA73775.1          GDRTAFRNMVNTCHAAGVKVVADTVINHMS-----AGSGTGTGGSSYTKY
AEY88989.1          GDRTAFRNMVNTCHAAGVKVVVDTVINHMS-----AGSGTGTGGSSYTKY
BAF49593.1          GDRTAFRNMVNTCHAAGVKVVVDTVINHMS-----AGSGTGTGGSSYTKY
NP_827158.1         GDATAFQNMINTCHTAGVKVVVDTVVNHMS-----AGSGTGTGGSAYTKY
ZP_06916602.1       GDATAFRNMVSTCHAAGVKVVVDTVVNHMS-----AGSGTGTGGSSYSKY
YP_003492208        GDATAFKNMIDTCHAAGVKVVVDTVVNHMS-----AGSGTGTGGSSYTKY
YP_004816943        GDRTAFKNMIDTCHSAGVKVVADAVINHMS-----AGSGTGTGGSSYTKY
YP_004965987        GDRTAFKNMIDTCHAAGVKVVADAVINHMS-----AGSGTGTGGSSYTKY
ADZ45287            GNAQQFRAMVNTCSDAGVGVVVDSVINHMAAG-----SGTGTGGTAYTKY
ZP_09405139.1       GDRDSFAAMVESCHAAGVKVVADAVINHMA-----AGSGTGTGGTSYTKY
P08486.1|AMY_STRH   GDRDAFASMVSACHAAGVKVIADAVVNHMA-----AGSGR--HHAQYTKY
YP_004806014        GDREAFAGMVDACHSAGVKVMADAVVNHMA-----AGSGTGTGGTAYTKY
AAA82875.1          GDRDGFAAMVDACHSAGVKVVADAVINHMA-----AGSGTGTGGTAYTKY
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ZP_09178791.1       DYPGIWSGADMDDCRSEIND---YGNRANVQNCELVGLADLDTGEPYVRD
P30270.1|S.griseu   DYPGIWSGADMDDCRSEIND---YGNRANVQNCELVGLADLDTGEPYVRD
P09794.1|AMY_STRL   DYPGIWSGADMDDCRSEIND---YGNRANVQNCELVGLADLDTGESYVRD
ZP_06908805.1       NYPGLYSSYDMDNCTSQINN---YQDRGNVQNCELVGLADLDTGEEYVRG
YP_001826792.       DYPGLYSGADMDDCRSQIGNN--YNDRGNVQNCELVGLADLDTGEEYVRG
ZP_09403579.1       DYPGLYSGADMDDCRSQINN---YNDRGNVQNCELVGLADLDTGEDYVRG
ZP_04707862.1       DYPGLYSGSDMDDCRAEISN---YNDRANVQNCELVGLADLDTGEDYVRG
YP_004925530.       NYPGLYSANDMNDCQSQISN---YGDRANVQNCELVGLADLDTGEEYVRG
YP_004802096.       DYPGLYSVNDMNDCQSEINN---YGDRANVQNCELVGLADLDTGEEYVRG
NP_631084.1         DYPGLYSSNDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEDYVRG
ZP_06526938.1       DYPGLYSSNDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEDYVRG
CAB06622.1          DYPGLYSSNDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEAYVRG
ABZ81609.1          DYPGLYSSNDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEDYVRG
CAA73926.1          DYPGLYSSNDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEDYVRG
EHN78434.1          DYPGIYSSSDLDNCTSQINN---YGDRFNVQECELVGLADLDTGEDYVRG
ZP_07314518.1       DYPGLYSRSDFDNCTSQISN---YGDRFNVQECELVGLADLDTGEDHARG
CCA55176.1          DYPGLYSGADMDDCRATIST---YQDRGNVQNCELVQLPDLDTGEDHVRG
P22998.1|AMY_STRV   DYPGLYSGSDMDDCRATISN---YQDRANVQNCELVQLPDLDTGEDHVRG
ZP_06711822.1       DYPGLYSAADMDDCTSVISN---YADRWNVQHCELVGLPDLDTNEEYVRK
EHN79425.1          DYPGTYSGADMDDCRTDITD---YRDRWNVQHCEMGSLPDLDTGEEYVRK
CAL64868.1          DYPGLYSKSDMDDCMKDIDN---YGNSWNVHHCELSGLPDLDTGEEYVRK
ZP_05002170.1       GYPGLYSGADMDDCRATISN---YQDRANIQNCELVQLADLDTGEDYVRG
ZP_05006689.1       GYPGIYSGFDLDDCRSGIVN---YQDRWHVQHCELVGLADLDTGEEYVRG
AAA96317.1          DYPGLYSAPDFDDCTAEITD---YQDRWNVQHCELVGLADLDTGEEYVRQ
P27350.2|Strepto    DYPGLYSAPDFDDCTAEITD---YQDRWNVQHCELVGLADLDTGEEYVRQ
ADB89947.1          DYPGLYSVYDFDDCTSQVTN---YQDRWNVQHCELVGLADLDTGEEYVRK
ZP_06579441.1       DYPGLYSSYDFDTCRSEINN---YRDRWNVQNCELVGLADLDTGEEYVRR
ZP_07313341.1       NYPGLYSSYDFDNCTSQINN---YGDRWNVQNCELVGLADLDTGEDYVRG
ZP_07303280.1       DYPGLYSSYDFDDCTSRISN---YQDRWNVQHCELVGLADLDTGEDYVRR
ZP_08805833.1       NYPGLYSSFDFDDCTSQVTN---YQDRWNVQRCELVGLADLDTGEPYVRG
CAA73775.1          SYPGLYSSYDFDDSTSQVTN---YQDRWNVQHCELVGLADLDTGESYVRG
AEY88989.1          DYPGLYSVYDFDDCTSQVSN---YSDRWNVQHCELVGLADLDTGEEYVRK
BAF49593.1          DYPGLYSVYDFDDCTSQVSN---YSDRWNVQHCELVGLADLDTGEEYVRK
NP_827158.1         NYPGLYSSYDMDDCTATITD---YTNRANVQNCELVGLADLDTGEEYVRK
ZP_06916602.1       NYPGLYSSYDMDDCTSTISD---YTNRANVQNCELVGLADLDTGEDYVRA
YP_003492208        DYPGLYSSFDFDDCTATISD---YTNRANVQNCELVTLADLDTGEEYVRS
YP_004816943        DYPGIYQAQDMDDCTSQISN---YQDRWNVQHCELVGLADLDTGESYVRT
YP_004965987        TYPGIYQSQDMDDCTAQISN---YQDRWNVQRCELVGLADLDTGESYVRD
ADZ45287            DYPGLYGSQDFNDCRQDIAPGDYAGDRWRVQNCELVGLSDLDTGSGYVQQ
ZP_09405139.1       DYPGTFQDQDFHTCRKDIAN---YGDRGDVQNCELVGLADLDTGSDAVRT
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P08486.1|AMY_STRH  NYPGFYQDQTFHGCRKSISD---YTNRDDVQTCELVDLADLGTGSDYVRT
YP_004806014       DYPGYFQDGDFHTCRADITD---YTDRDDVQNCELVGLSDLDTGKEEVRA
AAA82875.1         DYPGYFGDADFHTCRTAIKD---YTDRGDVQNCELVGLSDLDTGKDEVRS
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ZP_09178791.1      RIAAYLNDLLSLGVDGFRIDAAKHMPAADLTAIKAKVGNGSTYWKQEAIH
P30270.1|S.griseu  RIAAYLNDLLLLGVDGFRIDAAKHMPAADLTAIKAKVGNGSTYWKQEAIH
P09794.1|AMY_STRL  RIAAYLNDLLSLGVDGFRIDAAKHMPAADLTAIKAKVGNGSTYWKQEAIH
ZP_06908805.1      RIAGYLNDLLTLGVDGFRIDASKHMPADDLAAIKGKLTNPGIYWKHEAIH
YP_001826792.      RIAGYLNDLLSLGVDGFRIDAAKHMPAGDLANIKSRLTNPNVYWKHEAIY
ZP_09403579.1      KIAGYLNDLLSLGVDGFRIDAAKHMPAGDLANIKSRLSNPNVYWKHEAIH
ZP_04707862.1      KIAGYLNDLLSLGVDGFRIDAAKHMPAADLANIKSRLTSPNVYWKQEAIY
YP_004925530.      KIAGYLNDLLSLGVDGFRIDAAKHMPAADLANIKSRLSNPGVYWKHEAIY
YP_004802096.      KIAGYLNDLLSLGVDGFRIDAAKHMPAGDLANIKSRLSNPNVYWKQEAIY
NP_631084.1        KIAGYLNDLLSLGVDGFRIDAAKHMAAADLAAIKSRLSNPNVYWKHEAIY
ZP_06526938.1      KIAGYLNDLLSLGVDGFRIDAAKHMAAADLAAIKSRLSNPNVYWKHEAIY
CAB06622.1         KIAGYLTDLLSLGVDGFRIDAAKHMAAADLAAIKSRLSNPNVYWKHEAIY
ABZ81609.1         KIAGYLNDLLSLGVDGFRIDAAKHMAAADLAAIKSRLSNPNVYWKHEAIY
CAA73926.1         KIAGYLNDLLSLGVDGFRIDAAKHMAAPDLAAIKSRLSNPNVYWKHEAIY
EHN78434.1         KIAGYLNDLLSLGVDGFRIDAAKHMAAADLANIKSRLTNPNVYWKHEAIY
ZP_07314518.1      KIAGYLNDLLSLGVDGFRIDAAKHMAAGDLADIKSRLSNPDVYWKHEAIH
CCA55176.1         KIAGYLNDLLSLGVDGFRVDAAKHMPAADLAAIKARLSNPGAYWKQEAIF
P22998.1|AMY_STRV  KIAGYLNDLASLGVDGFRIDAAKHMPAADLANIKSRLTNPNVFWKLEAIH
ZP_06711822.1      TIAGYLNDLLSLGVDGFRIDAAKHIAEDDLANIKSRLTDPGVYWKQETIG
EHN79425.1         TIAGYLNDLLSLGVDGFRVDAAKHIAVDDLKDIKSRLTDPNAYMKLETIG
CAL64868.1         TIAGYLNDLLSLGVDGFRIDAAKHIPPADLADIKGRLRDPGVYWKQETIG
ZP_05002170.1      RIAGYLNDLLSLGVDGFRIDAAKHIPAADLANIKSRLNSPGAYWKQEAIY
ZP_05006689.1      RIAAYLNDLLSLGVDGFRVDAAKHMPAADLANIKSRLTRPDAYWKQEAIH
AAA96317.1         TIAGYMNDLLSLGVDGFRIDAAKHIPAEDLANIKSRLSNPNAYWKQEVIY
P27350.2|Strepto   TIAGYMNDLLSLGVDGFRIDAATHIPAEDLANIKSRLSNPNAYWKQEVIY
ADB89947.1         TIAGYLNDLLSLGVDGFRIDAAKHIPAEDLANIKSRLSDPHAYWKQEVIY
ZP_06579441.1      TIAGYMNDLLSLGVDGFRIDAAKHMPAADLANIKSRLTNPGAYWKQEVIY
ZP_07313341.1      AIAGYMNDLLSLGVDGFRVDAAKHMPAADLANIKSRLSNPSVYWKQEVIH
ZP_07303280.1      TIAGYMNDLLSLGVDGFRIDAAKHMDAADLANIKSRLSNPSVYWKQEVIY
ZP_08805833.1      AIAGYMNDLLSLGVDGFRIDAAKHMDAGDLADIKSRLTNPNAYWKQEVIY
CAA73775.1         GIARYMNDLLSLGVDGFRIDAAKHIDAADLANIKSRLTNPSVYWKQEVIY
AEY88989.1         TIAGYMNDLLTLGVDGFRIDAAKHIPAGDLANIKSRLTNPSAYWKQEVIY
BAF49593.1         TIAGYMNDLLTLGVDGFRIDAAKHIPAGDLANIKSRLTNPSAYWKQEVIY
NP_827158.1        TIAGYMNTLLGYGADGFRVDAVKHIPAADLANIKSRLTNPSVYWKQEVIY
ZP_06916602.1      TIAGYLNSLLGYGVDGFRIDAAKHIPATDLANIKSRLSNPSAYWKQEVIY
YP_003492208       AIAGYMNSLLGYGVDGFRIDAAKHIPAADLANIKSRLTNSSVYWKQEAIH
YP_004816943       RIAQYLNDLLSLGVDGFRVDGAKHIPTEDLAAIKSQLSDPGVYWKQEVIY
YP_004965987       RIAQYLNDLLSLGVDGFRIDAAKHMAAEDLAAIKSKLSNPNVYWKQEAIY
ADZ45287           TIADYMNTLLDWGVAGFRVDAAKHIPAQHLEQIRARVGDGSVYWKQEMIY
ZP_09405139.1      TIAGYLSDLRSLGVDGFRVDAAKHMSADDVAAIKGKMSDP-GFWVTEVIH
P08486.1|AMY_STRH  TIAGYLG-LRSLGVDGFRIDAAKHISATDLAAVKGKMKDP-GFWVQEVIY
YP_004806014       TIAAYLDDLRSLGVDGFRIDAAKHMAADDLAAVKEKMSDP-GFWVSEVIH
AAA82875.1         TIAAYLDGLRSMGVDGFRIDAAKHMAADDVAIQGQDERPP-GSGSPEVIQ
                    ** *:  *   *. ***:*. .*:   .:                * *

ZP_09178791.1      GAGEAVQPSEYLGTGDVQEFRYARD-LKRVFQNENLAHLKNFGEDWGYMA
P30270.1|S.griseu  GAGEAVQPSEYLGTGDVQEFRYARD-LKRVFQNENLAHLKNFGEDWGYMA
P09794.1|AMY_STRL  GAGEAVQPSEYLGTGDVQEFRYARD-LKRVFQNENLAHLKNFGEDWGYMA
ZP_06908805.1      GAGEAVSPSEYLGSGDVQEFRYGRG-LKQVFTGGNLTSLKNFGEGWGFME
YP_001826792.      GAGEAVSPSEYLGSGDVQEFRYGRS-LKQVFLNENLAHLKNFGEGWGFME
ZP_09403579.1      GAGEAVSPSEYLGSGDVQEFRYGRS-LKQVFLNENLAHLKNFGEGWGFME
ZP_04707862.1      GAGEAVSPSEYLGTGDVQEFRYGRS-LKQVFLNENLAHLKNFGEAWGFME
YP_004925530.      GAGEAVSPSEYLGSGDVQEFRYGRG-LKQVFNNENLAYLKNFGEGWGFME
YP_004802096.      GANEAVSPTEYLGTGDVQEFRYARG-LKQSFLGGNLADLKNFGEGWGFME
NP_631084.1        GAGEAVSPTEYVGSGDVQEFRYARD-LKRVFNGENLAYLKNFGEAWGHLP
ZP_06526938.1      GAGEAVSPTEYVGSGDVQEFRYARD-LKRVFNGENLAYLKNFGEAWGHLP
CAB06622.1         GAGEAVSPTEYVGSGDVQEFRYARD-LKRVFNGENLAYLKNFGEAWGHLP
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ABZ81609.1          AAGEAVSPTEYVGSGDVQEVRLRNANLTRVFNGENLAYLKNFGEAWGHLP
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EHN78434.1          GAGEAVSPAEYLGSGDVQEFRYARD-LKRVFNNENLAYLKNFGEAWGYLP
ZP_07314518.1       GAGEAVSPSEYLGSGDVQEFRYARD-LKRIFTGENLAYLKNFGESWGYMA
CCA55176.1          GAGEAVSPSEYLGNGDVQEFRYARD-LKRVLQNEKLAYLKNFGEAWGYMP
P22998.1|AMY_STRV   GAGEAVSPSEYLGSGDVQEFRYARD-LKRVLQGEKLSYLKNFGEAWGHMP
ZP_06711822.1       AAGEAVQPSEYYNTGDVQEFHYAYD-LKRVFTGEKLAELKNFGEAWGYVP
EHN79425.1          APGEAVQPSEYLGVGDPQEFTYAYD-LKRVFSGEKLAYLKNFGEDWGYLP
CAL64868.1          AYGEAVQPAQYTGTGDVQEFSYGRD-LKRVFSGEKLAYLKNLGEAWGYVA
ZP_05002170.1       GAGEAVSPGEYLGNGDVQEFRYARD-LKRVFQSEDLAHLKNFGEAWGYLP
ZP_05006689.1       GEGEPIAPGEYLGTGDVQEFRYARD-LKRVFHGEKLAYLRNYGEGWGYLP
AAA96317.1          GAGEAVQPGEYTGTGDVQEFRYAYD-LKRVFTQEHLAYLKNYGEDWGYLS
P27350.2|Strepto    GAGEPPKPGEYTGTGDVQEFRYAYD-LKRVFTQEHLAYLKNYGEDWGYLS
ADB89947.1          GAGEAVQPGEYTGNGDVQEFRYAYD-LKRVFNDENLAYLKNYGEGWGYLS
ZP_06579441.1       GAGEAVQPTEYTGNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYMN
ZP_07313341.1       GAGEAVQPTEYTGNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYLN
ZP_07303280.1       GSGEAVQPTEYTSNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYMN
ZP_08805833.1       GTGEAVQPAEYTRNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYLG
CAA73775.1          GAGEAVQPTEYTGNGDVQEFPYAYD-LKRVFNNENLAYLKNYGEAWGYLN
AEY88989.1          GAGEAVQPSEYTGNGDVQEFRYAYD-LKRVFTSEKLAYLTNYGEGWGYLN
BAF49593.1          GAGEAVQPSEYTGNGDVQEFRYAYD-LKRVFTSEKLAYLTNYGEGWGYLN
NP_827158.1         ASGEAVQPTEYTGNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYLN
ZP_06916602.1       GSGEAVQPTEYTGNGDVQEFRYAYD-LKRVFNNENLAYLKNYGEGWGYLS
YP_003492208        GSGEAVQPGEYTGNGDVQEFRYAFD-LKRVFTNENLAYLKNYGEGWGYMN
YP_004816943        GEGEAVSPTEYLRNGDVQEFRYGRD-LKRVFQGEKLANLKNFGEGWGYMA
YP_004965987        GAGEAVSPTEYLGNGDVQEFRYGRD-LKRVFTSEKLAYLNNFGEGWGYMA
ADZ45287            GAGEAIHPDEYTGAGDVQEFRHAFD-IKRIFQNERLAYLDDFGQSWGYLP
ZP_09405139.1       GGGEAVQPEEYTSIGDVDEFRYGGH-LKSAFQGGGLPGLKSIADGK--LA
P08486.1|AMY_STRH   GAGEAVRPDEYTGIGDVDEFRYGTH-LKSAFQSGNIAQLKSVADGK--LW
YP_004806014        GGGEAVRPEEYTGVGDVDEFRYGGH-LRSAFQGGDIAQLKSVADGK--LG
AAA82875.1          GGGEAVQPEEYTEIGDVDEFRYGGH-LKSAFQGGGIAQLKAVADRK--LG
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ZP_09178791.1       SGKSAV FVDNHDTE RGGDTLNYKNGSAYTLAGVFMLAWPYGSPDVHSGYE
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P09794.1|AMY_STRL   SGKSAV FVDNHDTE RGGDTLNYKNGSAYTLAGVFMLAWPYGSPDVHSGYE
ZP_06908805.1       SGRSAV FVANHDTE RGGDTLTYKDGANYTLAHVFMLAWPYGSPDVHSGYE
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ZP_09403579.1       SGKSAV FVDNHDTE RGGDTLNYKNGSAYTLASVFMLAYPYGSPDVHSGYE
ZP_04707862.1       SGKSGV FVDNHDTE RNGETLTYKNGSAYTLANVFMLAHPYGSPDVHSGYE
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YP_004802096.       SGRSAV FVDNHDTE RNGETLNYKNGADYTLASVFMLAYPYGSPDVHSGYE
NP_631084.1         SDEAAV FVTNHDTE RNGETLTYKDGATYTLAHVFMLAWPYGSPDVHSGYE
ZP_06526938.1       SDEAAV FVTNHDTE RNGETLTYKDGATYTLAHVFMLAWPYGSPDVHSGYE
CAB06622.1          SDEAAV FVTNHDTE RNGETLTYKDGATYTLAHVFMLAWPYGSPDVHSGYE
ABZ81609.1          SDEAGV FVNKHDTE RNGETLTYKDGATYTLAHVFMLAWPYGSPDVHSGYE
CAA73926.1          SDEAAV FVTNHDTE RNGETLTYKDGATYTLAHVFMLAWPYGSPDVHSGYE
EHN78434.1          SDQAAV FVTNHDTE RNGETLTYKDGANYTLAHVFMLAWPYGSPDVHSGYE
ZP_07314518.1       SDKSNV FVANHDTE RGGDTLSYRDGAGYTLAHVFMLAWPYGSPDVHSGYE
CCA55176.1          SAQSGV FVDNHDTE RGGDTLSYKDGANYTLASVFMLAWPYGSPDVHSGYE
P22998.1|AMY_STRV   SGQSGV FVDNHDTE RGGDTLSYKDGANYTLASVFMLAWPYGSPDVHSGYE
ZP_06711822.1       SGKASV FVTNHDTE RNGATLTYKNGAAYTLAHVFMLAWPYGSPDVHSGYE
EHN79425.1          SDKASV FVTNHDTE RNGRTLTYKDGATYTLAQVFSLAWPYGAPDVHSGYS
CAL64868.1          GDKAAT FVTNHDTE RHNATLTYKDGAKYTLAHVFMLAWPYGSPDVHSGYQ
ZP_05002170.1       SAQAGV FVDNHDTE RGGDTLNYKDGSAYTLASVFALAWPYGSPDVHSGYE
ZP_05006689.1       SDRAGV FVDNHDTE RLGDTLNYQDGALYTLANVFMLAWPYGAPDVHSGYE
AAA96317.1          STTAGV FVDNHDTE RNGSTLNYKNDATYTLANVFMLAWPYGAPDINSGYE
P27350.2|Strepto    STTAGV FVDNHDTE RNGSTLNYKNDATYTLANVFMLAWPYGAPDINSGYE
ADB89947.1          SSVAGV FVDNHDTE RNGSTLNYKDGANYTLANVFMLAWPYGAPDVNSGYE
ZP_06579441.1       GSVSGV FVDNHDTE RNGSTLSYKDNANYTLANVFMLAWPYGAPDINSGYE
ZP_07313341.1       SGVSGV FVDNHDTE RNGSTLSYKDNANYTLANVFMLAWPYGAPDINSGYE
ZP_07303280.1       SGVSGV FVDNHDTE RNGSTLSYKDNANYTLANVFMLAWPYGAPDINSGYE
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ZP_08805833.1      SGVAGV FVDNHDTE RNGSTLNYKDNASYTLANVFMLAWPYGAPDINSGYE
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AEY88989.1         SSVAGV FVDNHDTE RNGSTLNYKSGADYTLANVFMLAWPYGAPDVNSGYE
BAF49593.1         SSVAGV FVDNHDTE RNGSTLNYKSGADYTLANVFMLAWPYGAPDVNSGYE
NP_827158.1        SSVAGV FVDNHDTE RNGSTLNYKDGANYTLANVFMLAYPYGAPDINSGYE
ZP_06916602.1      SSVAGV FVDNHDTE RNGSTLNYKDGADYTLANVFMLAYPYGAPDINSGYE
YP_003492208       SSVAGV FVDNHDTE RNGSTLSYKDNATYTLANVFMLAYPYGAPDINSGYE
YP_004816943       SGQSGV FVDNWDTE RNGSTLTYKNGADYTLANVFMLAWPYGSPDVHSGYE
YP_004965987       SGKSGV FVDNWDTE RNGSTLSYKDNANYTLANVFMLAWPYGSPDVHSGYE
ADZ45287           SAGAGV FVDNHDTE RNGSTLTYRDGSAYTLANVFMLAWNYGSPDIHSGYE
ZP_09405139.1      GASART FVDNWDTE RNGSTLTHKDGAAYTLANVFMLASPYGSPNVFSGYT
P08486.1|AMY_STRH  QRQART FVDNWDTE RNGSTLTYKDGAAYTLANVFMLASPYGSPNVYSGYE
YP_004806014       SDSART FVDNWDTE RNGSTLTYKDGAAYTLANVFMLASPYGSPNVYSGYE
AAA82875.1         SASART FVDNWDTE RNGSTLTYKDGAAYTLANVFMLASPYGSPNVYSGYE
                       : .** : **:  . **.::..: **** ** **  **:*:: ***

ZP_09178791.1      FTDHDAGPPNGGTVNACYSDGWKCQHAWPELSSMVGLRNTASGQPVTNWW
P30270.1|S.griseu  FTDHDAGPPNGGTVNACYSDGWKCQHAWPELSSMVGLRNTASGQPVTNWW
P09794.1|AMY_STRL  FTDHDAGPPNGGTVNACYSDGWKCQHAWPELSSMVGLRNTASGQPVTNWW
ZP_06908805.1      FTQHDAGPPNGGAVTACYSDGWKCQHSWREISSMVGLRNTARGQGVTNWW
YP_001826792.      FSDHDAGPPNGGQVNACYSDGWKCQHAWREISSMVGFRNTARGQSVGNWW
ZP_09403579.1      FSDHDAGPPNGGQVNACYSDGWKCQHAWREISSMVGFRNTARGQGVTNWW
ZP_04707862.1      FSDYAAGPPGGGQVNACYSDGWKCQHAWREISSMVGFRNAARGQGVTNWW
YP_004925530.      WSDKDAGPPNGGQVNACYSDGWKCQHAWREISSMVGFRNTARGQAVTNWW
YP_004802096.      WSDKDAGPPSGGQVNACYSDGWKCQHAWTEISSMVGFRNAARGQAVTNWW
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CAB06622.1         FTDHDAGPPNGGQVNACYSDGWKCQHAWREISSMVAFRNTARGQGVTDWW
ABZ81609.1         FTDHDAGPPNGGQVNACYSDGWKCQHAWREISSMVAFRNTARGQGVTDWW
CAA73926.1         FTDHDAGPPNGVQVNACYSDGWKCQHAWCEISSMVAFRNTARGQGVTDWW
EHN78434.1         FTDHDAGPPNGGQVNACHSDGWKCQHTWREISSMVAFRNTARGQAVTDWW
ZP_07314518.1      YTDRDAGPPNGGQVNACYSDGWKCQHAWREISSMVGFRNAARGQAVTDWW
CCA55176.1         WTDKDAGPPSGGQVNACYTDGWKCQHAWREISSMVAFRNTARGQAVTGWW
P22998.1|AMY_STRV  WTDKDAGPPNNGQVNACYTDGWKCQHAWREISSMVAFRNTARGQAVTNWW
ZP_06711822.1      FTDNDAAGPDNGQVGACYTDGWKCQHAWREIASMVKFRNTAAGTAVTDWW
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ZP_05002170.1      WSDKDAGPPNGGTVKACYADGWKCQHAWREISSMVGFRNAARGQSVTNWW
ZP_05006689.1      WTDKDAGPPNGGRVDACWQDGWKCQHAWPEIASMVGFRNAARGEPVTHWW
AAA96317.1         WSDPDAGPPDGGHVDACWQNGWKCQHKWPEIASMVAFRNATRGEPVTDWW
P27350.2|Strepto   WSDPDARPPDGGHVDACWQNGWKCQHKWPEIASMVAFRNATRGEPVTDWW
ADB89947.1         WSDADAGPPNGGRVNACWQDGWKCQHKWPEIESMVAFRNTTRGEPVTNWW
ZP_06579441.1      FSDHDAGPPNGGQVNACWQNGWKCQHAWPEIKSMVAFRNATRGQAVTNWW
ZP_07313341.1      FSDQDAGPPNGGRVDACWQNGWKCQHAWPEIKSMVAFRNATRGQSVTNWW
ZP_07303280.1      FSDPDAGPPGGGQVNACWQDGWKCQHNWPEIKSMVGFRNATRRQAVTNWW
ZP_08805833.1      WSDADAGPPNGGSVGACWQNGWKCQHAWPEIRSMVGFRNATRGQAVTDWW
CAA73775.1         WSDAAAGPPN-ATVNACWQDGWKCQHKWPEIIRMVGFRNATRGQAVTDWW
AEY88989.1         WSDADAGPPNGGQVNACWQDGWKCQHAWPEIKSMVAFRNATRGQAVTNWW
BAF49593.1         WSDADAGPPNGGQVNACWQDGWKCQHAWPEIKSMVAFRNATRGQAVANWW
NP_827158.1        WSDADAGPPGGGTVNACWQDGWKCQHAWPEIKAMVAFRNATRGESVTNWW
ZP_06916602.1      WSDKDAGPPNSGSVNACWQDGWKCQHAWPEILRMVAFRNAVRGESVTNWW
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YP_004816943       FGDNDAGPPNGGTVGACYQDGWKCQHKWPEIMSMVALRNTARGASVTNWW
YP_004965987       FSDNDAGPPNGGTVNACWQDGWKCQHKWPEIIKMVAFRNTARGATVTNWW
ADZ45287           FTDYDAGPPRGGEVRDCYQDNWKCQHAWPEIASMVGFRNAVGDAAVTNWW
ZP_09405139.1      WTDKDAGPPNGGAAD-CGSGAWTCTHAQQAVTGMVGFHNAVAGAELTDWW
P08486.1|AMY_STRH  WTDKDAAAG--------GSTGWTDDAAKREITGMVGFRNAVGSAELTNWW
YP_004806014       WSSEDAGPPS-------GADGWTNVHARRAITGMVGFRNTVGSAELTDWW
AAA82875.1         WSDADAGPPS-------GADGWTDTHAQQTITGLVGFRNAVGSAELTDWW
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ZP_09178791.1      DNGGDQIAFGRGDKAYVAINHEGSALNRTFQSGLPGGAYCDVQSGRSVTV
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P09794.1|AMY_STRL  DNGGDQIAFGRGDKAYVAINHEGSALNRTFQSGLPGGAYCDVQSGRSVTV
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ZP_09403579.1      DNGGDQIAFGRGNKAYVAINHEGSALNRTFQTSLPAGDYCDVQSGKGVHV
ZP_04707862.1      DNGGDQIAFGRGTKAYVAINHEGSALNRTFQTSLPAGDYCDVQSGNGVTV
YP_004925530.      DNGGDQIAFGRGSKAYVAINHEGSSLSRTFQTSLPAGDYCDVQSGKGVTV
YP_004802096.      DNGGDQIAFGRGSKAYVAINHEGSSLTRTFQTSLPAGDYCDVQSGKGVTV
NP_631084.1        DNGGDQIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQTGKGVTV
ZP_06526938.1      DNGGDQIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQTGKGVTV
CAB06622.1         DNGGDQIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQTGKGVTV
ABZ81609.1         DNGGDQIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQTGKGVTV
CAA73926.1         DNGGDQIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQTGKGVTV
EHN78434.1         DNGGDRIAFGRGSKAYVAINHEGTSLTRTFQTSLPAGDYCDVQSGKGVTV
ZP_07314518.1      DNGGDQIAFGRGSKAYVALNHEGSSLTRTFQTSLPAGDYCDVQSGKGVTV
CCA55176.1         DNGNNAIAFGRGSKAYVAINHETSALTRTYQTSLPAGTYCDVQSNTPVTV
P22998.1|AMY_STRV  DNGNNAIAFGRGSKAYVAINHETSALTRTFQTSLPAGSYCDVQSNTPVTV
ZP_06711822.1      DNGGNQIAFGRGDKAYVAINHEGSSLTRTFQTSLPAGDYCDVQSGKGVTV
EHN79425.1         DNGNNQIAFGRGSKAYVAINHEGGSLTRTFQTSLPAGDYCDVQSGKGVTV
CAL64868.1         DNGSNAIAFGRGSKAYVAINHESGSLTRTFQTSLPAGTYCDVQSGKPVTV
ZP_05002170.1      DNGGDQIAFGRGTKAYVAINHEGSALTRTFQSSLPGGDYCDVQSGRTVTV
ZP_05006689.1      DNGDDRIAFGRGTKAYVAINREAQPLNREFQTGLAAGVYCDVQSRRSVTV
AAA96317.1         DDGADAIAFGRGSKGFVAINHESATVQRTYQTSLPAGTYCDVQSNTTVTV
P27350.2|Strepto   DDGADAIAFGRGSKGFVAINHESATVQRTYQTSLPAGTYCDVQSNTTVTV
ADB89947.1         DDGADAIAFGRGTKGFVAINHEPYTVSRTYQTSLPPGTYCDVQRNTTVTV
ZP_06579441.1      DNGGDAIAFGRGTKGFVAINHESGSLTRTYQTSLPAGTYCNVQNNTTVTV
ZP_07313341.1      DNGGDAIAFGRGSKGFVAINHESGSLTRTYQTSLAAGTYCNVQNNTTVTV
ZP_07303280.1      DNGGDAIAFGRGSKGFVAINHESGSLTRTYQTSLPAGSYCNVQNNTTVTV
ZP_08805833.1      DNGGDAIAFGRGSRGFVAINHESAQVSRTYQTSLPAGTYCNVQNNTTVSV
CAA73775.1         DNGGDAIAFGRGSKGFVAINHESATISRTYQTSLPPGPYCNVQNNTTCSV
AEY88989.1         DNGNNAIAFGRGTKGYVAINHESSSLTRTYQTSLPAGTYCNVQNNTPVTV
BAF49593.1         DNGNNAIAFGRGTKGYVAINHESSSLTRTYQTSLPAGTYCNVQNNTPVTV
NP_827158.1        DNGGDAIAFGRGAKGYVAINHESGSLTRTYQTSLTAGTYCNVQNNTGVTV
ZP_06916602.1      DNGGDAIAFGRGAKGYVAINHESSSLSRTYQTSLPAGTYCDVQHNTSVTV
YP_003492208       DNGSDAIAFGRGGKGFVAINHESSGLSRTYRTSLAAGTYCNVQNNTTVTV
YP_004816943       DNGNNAIAFGRGDKAYVAINHESGSLTQTFQTSLPAGGYCDVQSGKAVTV
YP_004965987       DNGNNAIAFGRGEKAYVAINHEDTSLTRTFQTSLPAGGYCDVQSGRSVTV
ADZ45287           DNGNDVIAFGRGDRGFVVVNHESAAVDRTWQSSLPGGTYCDVQSGRAVTV
ZP_09405139.1      DDGSSALAFARAGKGFVAVNNGDAELNRTFTTTLPAGTYCNVVAAAPDSC
P08486.1|AMY_STRH  DNGGRPLAFARSDKGFVALNNGDAALTQTFATSLPAGTYCDVVHAASSCD
YP_004806014       DNGGSAIAFGRGDKGFVALNNGDGDLSETLTTSLPAGTYCDVAVASPDDC
AAA82875.1         DNGGSALAFGRGDKGFVALNNADDALTETFTTSLPAGTYCNVAAASPDDC
                   *:*   :**.*. :.:*.:*.    : .   : *. * **:*

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P30270.1|S.griseu  GSDGTF------------------TATVAAGTALALHTGARTCSGGG--
P09794.1|AMY_STRL  GSDGTF------------------TATVAAGTALALHTGARTCSGGG--
ZP_06908805.1      DGSGRF------------------TATLGAGTALALHAGARDCGGGS--
YP_001826792.      NGSGQF------------------TATVPAGTAVALHTGARTCGGGG-S
ZP_09403579.1      NGSGQF------------------TATVPAGTAVALHAGARTCGGGG-T
ZP_04707862.1      NGSGQF------------------TATVPAGTAVALHVNARTCSGGGGT
YP_004925530.      NSSGQF------------------TATLGSNTAVALHVGARTCTGGG--
YP_004802096.      NGSGQF------------------TATLGSNTAVALHTGARTCTGGG--
NP_631084.1        DGAGRF------------------TATLGAGTAVALHVGARTCDGG---
ZP_06526938.1      DGAGRF------------------TATLGAGTAVALHVGARTCDGG---
CAB06622.1         DGAGRF------------------TATLGAGTAVALHVGARTCDGG---
ABZ81609.1         DGAGRF------------------TATLGAGTAVALHVGARTCDGG---
CAA73926.1         NGAGRF------------------TATLGAGTAVALHVGARTCDGG---
EHN78434.1         DGAGRF------------------TATLGANTAVALHVGARTCDGGG--
ZP_07314518.1      DGSGRF------------------TATLGAGTALALHAGARNCDGG---
CCA55176.1         NGSGQF------------------TATLGAHTALALHVGAMNCGSG--T
P22998.1|AMY_STRV  NSSGQF------------------TATLAANTAVALHVNATGCGS---T
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EHN79425.1         NSAGQF-------------------TATLGANTALALHVGAKTCGG---G
CAL64868.1         NGSGQF-------------------TATLGPDTALALHTGATSCTDP--G
ZP_05002170.1      GSGGQF-------------------TATLGAGTALALHTGARTCAGTP--
ZP_05006689.1      DTAGRF-------------------TASLAPNTALALHTAKRSCA-----
AAA96317.1         DSAGRF-------------------TAALGPDTALALHTGRTSC------
P27350.2|Strepto   DSAGRF-------------------TAALGPDTALALHNGRTSC------
ADB89947.1         NSSGQF-------------------TAALGPNTALALRAGKTRC------
ZP_06579441.1      NGSGQL-------------------TATLGSNTALAVYAGKSSC------
ZP_07313341.1      NGSGQL-------------------TATLGGNTALAVYAGKSGC------
ZP_07303280.1      SSSGQF-------------------TATLGANTALALQAGRPTC------
ZP_08805833.1      NSSGRL-------------------TATLGSGTALAVYAGKSSC------
CAA73775.1         NGSGQL-------------------TATLAANTALAVYAGKSSC------
AEY88989.1         NSSGQF-------------------TATLGSNTALALYAGKSSC------
BAF49593.1         NSSGQS-------------------TATLGSNTALALYAGKSSC------
NP_827158.1        DSSGRF-------------------TATLGANTALALYSGKSTC------
ZP_06916602.1      GSNGQF-------------------TATLGANTALAIYAGKTGC------
YP_003492208       NGSGQF-------------------TATLGAHTALALYAGKSSC------
YP_004816943       DSAGKF-------------------TATLGANTAVALHVGARNCG-----
YP_004965987       DSSGQF-------------------TATLGANTAVALHVGARSCG-----
ADZ45287           DGSGRF-------------------TASVAAGTALALHAGARDCSGNGGP
ZP_09405139.1      DGNATT------------VADDGTATVTVPARGAVALHT-----------
P08486.1|AMY_STRH  GDTVTVGDTEAQVDAAKSVALHVGATGQSACRQAVALHVPGQSAGSPRSS
YP_004806014       DGATVT------------VGDDGTVQASLPAHGALALHTGARTD------
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P09794.1|AMY_STRL  ---TGPGTGQTS-ASFHVNATTAWGENIYVTGDQAALGNWDPARALKLDP
ZP_06908805.1      ---TDPGPVSAG-ASFAVNATTVVGQNIYVTGNQPALGNWNPAGAVKLDP
YP_001826792.      NPDPGPGNGTSG-ASFGVNATTQLGQNIHVTGDQAALGNWNPATAPKLDP
ZP_09403579.1      NPDPDPGNGASG-ASFGVSATTQPGQNIHVTGDRTALGNWNPANAPKLDP
ZP_04707862.1      NPDPGPGNGSSG-ASFGVNATTQPGQNIHVTGDQSALGNWNPANAPKLDP
YP_004925530.      -TTPDPGTGQSG-ASFGVNATTQVGQNIYVTGSQAALGSWAPGSALKLDP
YP_004802096.      -TTPDPGTGGSG-ASFGVAATTVLGQNIYVTGDQATLGDWNPGGALKLDP
NP_631084.1        -DPGDPDPVSSG-VSFAVDATTSWGQNIYVTGNRPELGN-----------
ZP_06526938.1      -DPGDPDPVSSG-VSFAVDATTSWGQNIYVTGNRPELGNWNPGGALQLDP
CAB06622.1         -DPGDPDPVSSG-VSFAVDATTSWGQDIYVTGNRPGLGHWDPGGGLQLDP
ABZ81609.1         -DPGDPDPVSSG-VSFAVDATTSWGQNIYVTGNRPELGN-----------
CAA73926.1         -DPGDPDPVSFRRVPSAVDATTSWGQNIYVTGNRPELGNWNPGGALQLDP
EHN78434.1         -DPGDPDPVSSG-VSFAVDATTSWGQNIYVTGNRPELGNWNPAGALELDP
ZP_07314518.1      -TVPDPDPVASG-VSFAVNATTSWGQNIYVTGNRPELGNWNPGSALKLDP
CCA55176.1         TTPPPTTPPATPGASFNATATTVVGQDIYVTGNRAELGNWSPAAALKLDP
P22998.1|AMY_STRV  PTTPPTTPPATSGASFNVTATTVVGQNIYVTGNRAELGNWAPASALKLDP
ZP_06711822.1      TSGGDGGGSTATGASFNVNAATQWGENIYVTGNNAALGNWNTASALKLDP
EHN79425.1         STDPE----PAAGASFRVNATTSWGENIYVTGDNTRLGDWNPDNALKLDP
CAL64868.1         GGNPGENPGEDPGASFAVQAPTTWGENIYVTGNRSALGDWNPAGALKLDP
ZP_05002170.1      --------AGSAGAAFGVSATTVPGQNIYVTGDRAELGGWNTGSALKLDP
ZP_05006689.1      --------------------------------------------------
AAA96317.1         --------------------------------------------------
P27350.2|Strepto   --------------------------------------------------
ADB89947.1         --------------------------------------------------
ZP_06579441.1      --------------------------------------------------
ZP_07313341.1      --------------------------------------------------
ZP_07303280.1      --------------------------------------------------
ZP_08805833.1      --------------------------------------------------
CAA73775.1         ---------------------------------------------------
AEY88989.1         --------------------------------------------------
BAF49593.1         --------------------------------------------------
NP_827158.1        --------------------------------------------------
ZP_06916602.1      --------------------------------------------------
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YP_003492208       --------------------------------------------------
YP_004816943       --------------------------------------------------
YP_004965987       --------------------------------------------------
ADZ45287           --------------------------------------------------
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P08486.1|AMY_STRH  AKRVEQ-----------------------------------------------
YP_004806014       --------------------------------------------------
AAA82875.1         --------------------------------------------------


ZP_09178791.1      AAYPVWKLDVPLAAGTPFQYKYLRKDAAGKAVWESGANRTATVGTTGALT
P30270.1|S.griseu  AAYPVWKLDVPLAAGTPFQYKYLRKDAAGKAVWESGANRTATVGTTGALT
P09794.1|AMY_STRL  AAYPVWKLDVPLAAGTPFQYKYLRKDAAGKAVWESGANRTATVGTTGALT
ZP_06908805.1      AAYPVWKAEVNLAAGTSFEYKYIRKDGAGNVTWESGANRTATVPSSGKVA
YP_001826792.      AAYPVWKLDVNLPAGTTFAYKYVRKDAAGNVTWESGANRTATVPASGKVT
ZP_09403579.1      AAYPVWKLDVNLPAGTTFAYKYVRKDGSGNVTWESGANRTATVPASGKVT
ZP_04707862.1      ATYPVWKLDVALPAGTTFAYKYVRKDAAGNVTWESGANRTATVPASGKVT
YP_004925530.      ASYPVWKLDVALPAGTSFEYKYLRKDASGNVTWESGANRTATVPSSGKVA
YP_004802096.      AAYPVWKLDVALPAGTSFAYKYLRKDAAGNVTWESGANRTATVPSSGKVA
NP_631084.1        --------------------------------------------------
ZP_06526938.1      AAYPVWKRDVELPEGTTFEYKYLRKDDAGNVTWESGANRTATVNTTKTTL
CAB06622.1         AAYPVWKRDVELPEGTTFEYKSLRKADAGNVTWESGANRTATVNTTKTTL
ABZ81609.1         --------------------------------------------------
CAA73926.1         AAYPVWKRDVELPEGTTFEYKYLRKDDAGNVTWESGANRTATVNTTKTTL
EHN78434.1         AAYPVWKRDVELPEGTSFEYKYLRKDASGNVTWESGANRTATVGDTGTAL
ZP_07314518.1      AAYPVWKLDVELPEGTSFEYKYIRKDASGNVTWESGANRTATVTATRTAL
CCA55176.1         ATYPVWKLTVGLPAGTAFEYKYLRKDAAGNVTWESGANRTATVPASGRIV
P22998.1|AMY_STRV  ATYPVWKLTVGLPAGTSFEYKYIRKDAAGNVTWESGANRTATVPASGQLV
ZP_06711822.1      AGYPVWKLAVSLPAGTSFEYKYLRKDASGNVIWESGANRTATVGSDGLVT
EHN79425.1         AAYPVWKLDVSLPAGTSFEYKYLRKDANGKVTWESGANRTATVSSSGTVV
CAL64868.1         AAYPVWKLDVALPAGTSFEYKFLRKDASGKVTWESGPNRTATVPSSGRTL
ZP_05002170.1      AAYPVWKLDVTLPAGAAFSYKYLRKDAAGNVTWESGANRTASVPASGRVT
ZP_05006689.1      --------------------------------------------------
AAA96317.1         --------------------------------------------------
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ADB89947.1         --------------------------------------------------
ZP_06579441.1      --------------------------------------------------
ZP_07313341.1      --------------------------------------------------
ZP_07303280.1      --------------------------------------------------
ZP_08805833.1      --------------------------------------------------
CAA73775.1         --------------------------------------------------
AEY88989.1         --------------------------------------------------
BAF49593.1         --------------------------------------------------
NP_827158.1        --------------------------------------------------
ZP_06916602.1      --------------------------------------------------
YP_003492208       --------------------------------------------------
YP_004816943       --------------------------------------------------
YP_004965987       --------------------------------------------------
ADZ45287           --------------------------------------------------
ZP_09405139.1      --------------------------------------------------
P08486.1|AMY_STRH  --------------------------------------------------
YP_004806014       --------------------------------------------------
AAA82875.1         --------------------------------------------------


ZP_09178791.1      LNDTWRG-
P30270.1|S.griseu  LNDTWRG-
P09794.1|AMY_STRL  LNDTWRG-
ZP_06908805.1      LNDTWRN-
YP_001826792.      LTADVWRG
ZP_09403579.1      LTADVWRS
ZP_04707862.1      LTADVWRG
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YP_004925530.       LTADVWRS
YP_004802096.       LTSDVWRG
NP_631084.1         --------
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CAB06622.1          NDTWRN--
ABZ81609.1          --------
CAA73926.1          NDTWRN--
EHN78434.1          NDTWRD--
ZP_07314518.1       NDTWRN--
CCA55176.1          LNDTFRS-
P22998.1|AMY_STRV   LNDTFRS-
ZP_06711822.1       LNDTWRG-
EHN79425.1          LGDTWRD-
CAL64868.1          LSDTWRG-
ZP_05002170.1       LTDTWRG-
ZP_05006689.1       --------
AAA96317.1          --------
P27350.2|Strepto    --------
ADB89947.1          --------
ZP_06579441.1       --------
ZP_07313341.1       --------
ZP_07303280.1       --------
ZP_08805833.1       --------
CAA73775.1          --------
AEY88989.1          --------
BAF49593.1          --------
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YP_004816943        --------
YP_004965987        --------
ADZ45287            --------
ZP_09405139.1       --------
P08486.1|AMY_STRH   --------
YP_004806014        --------
AAA82875.1          --------
```
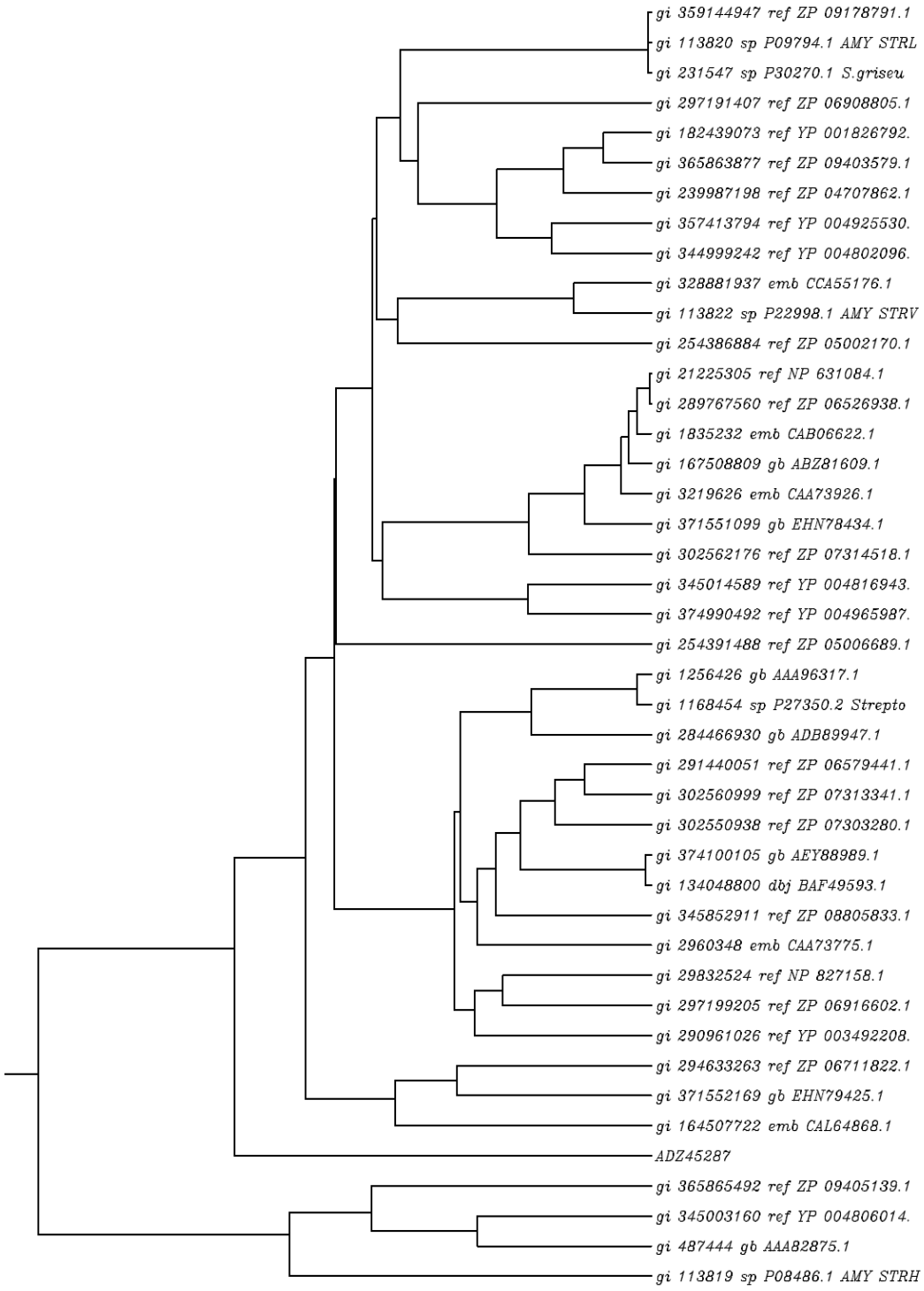

Fig. 5.14. ClustalW alignment of Amy Strplo 716 with the α-amylases of other Streptomyces. All the α-amylases are indicated by their GenBank accession numbers.

An UPGMA tree generated using the tree generation option of CLUSTALW shows one minor cluster having the α-amylases of four *Streptomyces* and a major cluster consisting of the α-amylases of several *Streptomyces* (Fig 5.15). Amy Strplo 716 forms a separate branch in this major cluster.

Fig 5.15. UPGMA tree generated by using CLUSTALW with Amy Strplo 716 and several *Streptomyces* α-amylases.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gi 113820 sp P09794.1 AMY STRL
gi 231547 sp P30270.1 S.griseu
gi 297191407 ref ZP 06908805.1
gi 182439073 ref YP 001826792.
gi 365863877 ref ZP 09403579.1
gi 239987198 ref ZP 04707862.1
gi 357413794 ref YP 004925530.
gi 344999242 ref YP 004802096.
gi 328881937 emb CCA55176.1
gi 113822 sp P22998.1 AMY STRV
gi 254386884 ref ZP 05002170.1
gi 21225305 ref NP 631084.1
gi 289767560 ref ZP 06526938.1
gi 1835232 emb CAB06622.1
gi 167508809 gb ABZ81609.1
gi 3219626 emb CAA73926.1
gi 371551099 gb EHN78434.1
gi 302562176 ref ZP 07314518.1
gi 345014589 ref YP 004816943.
gi 374990492 ref YP 004965987.
gi 254391488 ref ZP 05006689.1
gi 1256426 gb AAA96317.1
gi 1168454 sp P27350.2 Strepto
gi 284466930 gb ADB89947.1
gi 291440051 ref ZP 06579441.1
gi 302560999 ref ZP 07313341.1
gi 302550938 ref ZP 07303280.1
gi 374100105 gb AEY88989.1
gi 134048800 dbj BAF49593.1
gi 345852911 ref ZP 08805833.1
gi 2960348 emb CAA73775.1
gi 29832524 ref NP 827158.1
gi 297199205 ref ZP 06916602.1
gi 290961026 ref YP 003492208.
gi 294633263 ref ZP 06711822.1
gi 371552169 gb EHN79425.1
gi 164507722 emb CAL64868.1
ADZ45287
gi 365865492 ref ZP 09405139.1
gi 345003160 ref YP 004806014.
gi 487444 gb AAA82875.1
gi 113819 sp P08486.1 AMY STRH

The regions 3' upstream and 5' downstream are not necessarily conserved even in closely related organisms. The sequence analysis of the derived partial amino acid sequence is discussed below:

```
PPA                 QYAPQTQSGRTSIVHLFEWRWVDIALECERYLGPKGFGGVQVSPPNENIV 50
Amy Strplo 716      -------GEKDVTAVLFSWDFDSIARECTDRLGPAGYGFVQVSPPQEHIQ 43
                           . :   . **.* : .** **   *** *:* ******:*:*

PPA                 VTNPSRPWWERYQPVSYKLCTRSGNENEFRDMVTRCNNVGVRIYVDAVIN 100
Amy Strplo 716      GSQ----WWTQYQPVSYDIAGRLGNAQQFRAMVNTCSDAGVGVVVDSVIN 89
                     ::    ** :******.:. * ** ::** **. *.:.** : **:***

PPA                 HMCGSGAAAGTGTTCGSYCNPGNREFPAVPYSAWDFNDGKCKTASGGIES 150
Amy Strplo 716      HMAAGSGTGTGGTAYTKYDYPG-------LYGSQDFNDCRQDIAPG--DY 130
                    **.....:.  **:  .*  **        *.: **** : . *.*  :

PPA                 YNDPYQVRDCQLVGLLDLALEKDYVRSMIADYLNKLIDIGVAGFRIDASK 200
Amy Strplo 716      AGDRWRVQNCELVGLSDLDTGSGYVQQTIADYMNTLLDWGVAGFRVDAAK 180
                     .* ::*::*:**** **   ..**:. ****:*.*:* ******:**:*

PPA                 HMWPGDIKAVLDKLHNLNTNWFPAGSRPFIFQEVIDLGGEAIQSSEYFGN 250
Amy Strplo 716      HIPAQHLEQIRARVGDGSVYWK---------QEMIYGAGEAIHPDEYTGA 221
                    *: . .:: :  :: : .. *          **:*  .****:..** *

PPA                 GRVTEFKYGAKLGTVVRKWSGEKMSYLKNWGEGWGFMPSDRALVFVDNHD 300
Amy Strplo 716      GDVQEFRHAFDIK---RIFQNERLAYLDDFGQSWGYLPSAGAGVFVDNHD 268
                    * * **:. .:    * :..*:::**.::*:.**::**  * *******

PPA                 NQRGHGAGGASILTFWDARLYKVAVGFMLAHPYGFTRVMSSYRWARNFVN 350
Amy Strplo 716      TER-----NGSTLTYRDGSAYTLANVFMLAWNYGSPDIHSGYEFT----- 308
                    .:*     ..* **: *.  *.:*  ****  ** . : *.*.::

PPA                 GQDVNDWIGPPNNNGVIKEVTINADTTCGNDWVCEHRWRQIRNMVWFRNV 400
Amy Strplo 716      -----DYDAGPPRGGEVRDCYQ-------DNWKCQHAWPEIASMVGFRNA 346
                         *: . * ..* :::          ::* *:* * :* .** ***.


PPA                 VDGQPFANWWANGSNQVAFGRGNRGFIVFNNDDWQLSSTLQTGLPGGTYC 450
Amy Strplo 716      VGDAAVTNWWDNGNDVIAFGRGDRGFVVVNHESAAVDRTWQSSLPGGTYC 396
                    *.. ..:*** **.: :*****:***:*.*::.  :. * *:.*******

PPA                 DVISGDKVGNSCTGIKVYVSSDGTAQFSISNSAEDPFIAIHAESKL---- 496
Amy Strplo 716      DVQSGR-----------AVTVDGSGRFTAS-VAAGTALALHAGARDCSGN 434
                    ** **            *: **:.:*: *  * .. :*:** ::

PPA                 ---
Amy Strplo 716      GGP 437
```

Fig.5.16. Clustalw alignment of porcine pancreatic α-amylase (PPA: AF064742) with Amy Strplo 716 (ADZ45287).

Fig 5.16 shows the ClustalW alignment of Amy Strplo 716 with the amylase from porcine pancreas (AF064742). ADZ45287 shows almost 40% similarity with PPA. Enquist *et al* (1971) and Benigni *et al* (1975) have reported 36% similarity between *Streptomyces* and mammalian amylases. The four regions conserved in all the α-amylases are highlighted in Figs 5.14 and 5.16. The 'Phe-Glu-Trp' triplet (FEW) at the

amino-terminal end of the amylase has been suggested as a likely candidate for interaction with tendamistat, an inhibitor which inhibits mammalian and *Streptomyces* amylases (Long *et al*, 1987). All the *Streptomyces* α-amylases have either a histidine or a tryptophan in the third conserved region (represented by H 267 in Amy Strplo 716).

The sequence alignment of Amy Strplo 716 with PPA shows that the three active site residues Asp 177, Glu 204 and Asp 268 in ADZ45287 match with the three corresponding residues Asp 197, Glu 233 and Asp 300 of PPA. A comparison with PPA shows that residues in PPA ie H 101, H 201 and H 299 involved in substrate binding are also conserved in Amy Strplo 716 as H 90, H 181 and H 267. Similarly residues N 100 and D 167 in PPA involved in Ca^{2+} binding are conserved as N 89 and D 147 in Strplo Amy 716. However, R 158 in PPA involved in Ca^{2+} binding is replaced by Q 138. The significance of this replacement can be explained only on studying the crystal structure of this enzyme. R 195 and N 298 in PPA involved in Cl- binding are represented as R 175 and N 266 in Strplo Amy 716.

A glycine rich 'flexible loop' GHGAGG (residues 304-308 in PPA) which forms the surface edge of the substrate binding cleft in PPA, is absent in Amy Strlo 716. Residues E 20, S 303 and H 386 in ADZ45287 match with the E 27, S 340 and H and this triad has been shown to mimick the active site of proteases and lipases and was first described in the α-amylase from *Alteromonas haloplanctis*. Though there is no evidence for a catalytic role, this triad has been suggested to play an important role in stabilizing the structure of chloride dependent α-amylases. The disulfide bond C 70 – C 115 links domains A and B in PPA. This disulfide bond is absent in Amy Strlo 716 and also in the *Alteromonas haloplanctis* amylase (AHA) where it has been suggested that its absence in AHA provides a higher conformational freedom in the vicinity of the active site. This bridge is specific to mammalian Cl^- dependent amylases and is absent in insects, invertebrates and microorganisms where the physiological temperature is well below 37 $^{\circ}$C (Aghajari *et al*, 1998).

Melouli *et al* (1998) in their studies on the amylase from *Streptomyces* TO1 (Y13332) have suggested the importance of amino-acid replacements in four positions in determining whether the enzyme would be psychrophilic, mesophilic or thermophilic. These replacements are Lys to Arg, Ser to Ala/Thr, Gly to Ala and Ile to Val. Such replacements in the amylase of *Streptomyces sp* TO1 are responsible for its thermophilic nature. Of these four positions, arginine is present in position 279 in Strlo

Amy 716, the corresponding position being 302 in Y13332 Presence of R279 in Amy Strplo 716 could possibly explain the mesophilic nature (optimum temperature of 45^0C) of this enzyme.

The amino acid composition of Amy Strplo 716 is shown in Table 5.7. The percentage of hydrophobic residues (Ala, Val, Ile, Leu, Phe, Pro and Met) in Amy Strplo 716 is 34.78%. This value compares well with similar values of 34% (AHA: *Alteromonas haloplanctis* α-amylase), 35% (HPA: Human pancreatic α-amylase), 34% (HSA : Human salivary α-amylase) and 34% (PPA : Porcine pancreatic α-amylase). Hydrophobic interactions have been suggested to contribute to the thermostability of proteins (Aghajari *et al*, 1998). The proportion of Arg and Lys in Amy Streplo 716 is 5.49% and 1.37%. Shirai *et al* (2007) have suggested that an increase in Arg content with a simultaneous decrease in Lys content is an important factor which has contributed to the alkaline adaptation process. Their studies on the crystal structure of *Bacillus sp* KSM 1378 α-amylase (AmyK) revealed that a decrease in Lys resulted in loss of Lys-Asp/Glu ion pairs which was partly compensated by the acquisition of Arg-Asp/Glu ion pairs. However, the significance of the role of the above mentioned amino acids in Amy Strplo 716 can be deciphered only by a detailed study of its crystal structure.

Table 5.7. Amino acid composition of Amy Strplo 716

Amino acid			**No. of Residues**
Alanine	Ala	A	45 (10.30 %)
Cysteine	Cys	C	8 (1.83 %)
Aspartic acid	Asp	D	40 (9.15 %)
Glutamic acid	Glu	E	15 (3.43 %)
Phenylalanine	Phe	F	17 (3.89 %)
Glycine	Gly	G	56 (12.81 %)
Histidine	His	H	11 (2.52 %)
Isoleucine	Ile	I	15 (3.43 %)
Lysine	Lys	K	6 (1.37 %)
Leucine	Leu	L	19 (4.35 %)

Methionine	Met	M	6 (1.37 %)
Asparagine	Asn	N	18 (4.12 %)
Proline	Pro	P	15 (3.43 %)
Glutamine	Gln	Q	23 (5.26 %)
Arginine	Arg	R	24 (5.49 %)
Serine	Ser	S	26 (5.95 %)
Threonine	Thr	T	23 (5.26 %)
Valine	Val	V	35 (8.01 %)
Tryptophan	Trp	W	13 (2.97 %)
Tyrosine	Tyr	Y	22 (5.03 %)

The G+C content of the partial gene *Amy Strplo 716* is 70.38 mol %, which is in good agreement with the average G+C content of *Streptomyces* DNA (Gladek and Zakrzewska, 1984). The high G+C content results in an extremely biased usage of synonymous codons such that majority of the codons possessing G or C in the third position are preferred. There is also a preference of C over G in the third position in synonymous codons. This has been observed in *Streptomyces* genes (Hopwood *et al*, 1986; Malpartida, and Smith, 1986). A similar trend has also been observed in codon usage in ADZ45287. (Table 5.8).

Amino-acids represented by single letter code	**Codon**	**Frequency of codon usage per 1000.**	**Amino-acids represented by single letter code**	**Codon**	**Frequency of codon usage per 1000**
F	TTT	0.00	V	GTT	0.00
F	TTC	38.99	V	GTC	57.34
L	TTA	0.00	V	GTA	0.00
L	TTG	0.00	V	GTG	22.94
L	CTT	0.00	S	TCT	0.00

L	CTC	20.64	S	TCC	32.11
L	CTA	0.00	S	TCA	0.00
L	CTG	22.94	S	TCG	13.76
I	ATT	0.00	S	AGT	0.00
I	ATC	34.40	S	AGC	13.76
I	ATA	0.00	R	CGT	0.00
P	CCT	0.00	R	CGC	36.7
P	CCC	16.06	R	CGA	0.00
P	CCA	0.00	R	CGG	18.35
P	CCG	16.06	R	AGA	0.00
T	ACT	0.00	R	AGG	0.00
T	ACC	50.46	Y	TAT	0.00
T	ACA	0.00	Y	TAC	50.46
T	ACG	2.29	H	CAT	0.00
A	GCT	0.00	H	CAC	25.23
A	GCC	75.69	Q	CAA	0.00
A	GCA	0.00	Q	CAG	52.75
A	GCG	27.52	G	GGT	0.00
N	AAT	0.00	G	GGC	100.92
N	AAC	41.28	G	GGA	11.47
K	AAA	0.00	G	GGG	16.06
K	AAG	13.76	E	GAA	4.59
N	AAT	0.00	E	GAG	29.82
N	AAC	41.28	C	TGT	0.00
K	AAA	0.00	C	TGC	18.35
K	AAG	13.76	M	ATG	13.76

D	GAT	0.00	W	TGG	29.82
D	GAC	91.74			

Table 5.8: Codon Usage in ADZ45287, showing the frequency of codon usage per 1000.

Bioinformatics and Homology Modeling

An attempt was made to build a homology model of Amy Strplo 716. For homology modeling, it is necessary to first identify a suitable template. For this purpose, a BLAST search of the target amylase sequence Amy Strplo 716 against the Protein Data Bank was made to look for homologous proteins whose three-dimensional structures were available. The psychrophilic α-amylase from *Alteromonas haloplanctis* (PDB Id: 1B0I) was obtained as the best hit. The target sequence was found to have 48% sequence identity, over a query coverage of 96%, with the template 1B0I (Figure 5.17). The structure 1B0I has been determined at a resolution of 2.40 Angstrom. The sequence similarity along with the E-value of the target-template alignment (2e-135) shows higher confidence of using 1B0I as a template for model building.

Figure 5.17: Alignment of target sequence with template 1B0I

As the target (Amy Strplo 716) was an alkaline amylase, an attempt was also made to use the alkaline alpha-amylase from *Bacillus sp*. KSM-1378 (whose three-dimensional structure is deposited in PDB (PDB Id: 2DIE)) as a possible starting template structure for building the model of the target sequence. This template showed only 23% identity with the target sequence. The template and target had sequence lengths of 485 and 437 amino acids respectively. Although the overall homology seemed to be low, sequence conservation across N to C terminal was seen between the two sequences (Figure 5.18). 2DIE was also used as a possible starting template for model building, to crosscheck the overall folding of the target sequence.

```
Str             ---GEKDVTAVLFSWDFDS------IARECTDRLGPAGYGFVQVSPPQEHIQ----GSQW
2DIE_A          HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDAANLKSKGITAVWIPPAWKGTSQNDVGYGA
                   * :..    *.*.: .        *: : .* . *   * :.*. :  .    *

Str             WTQYQPVSYDIAG----RLGNAQQFRAMVNTCSDAGVGVVVDSVINHMAAGSGTGTG---
2DIE_A          YDLYDLGEFNQKGTVRTKYGTRSQLQGAVTSLKNNGIQVYGDVVMNHKGGADGTEMVNAV
                :  *:  .::  *    : *. .*::. *.: .: *: *  * *:** ....**

Str             ----------------GTAYTKYDYPGLYGSQ-DFNDCRQDIAPGDYAGDR------WRV
2DIE_A          EVNRSNRNQEISGEYTIEAWTKFDFPGRGNTHSNFKWRWYHFDGTDWDQSRQLQNKIYKF
                                 *:**:*:**  .:: :*:    .:   *:  .*      ::.

Str             QNCELVGLSDLDTGSG--------YVQQTIADYMNTLLDWG--------VAGFRVDAAKH
2DIE_A          RGTGKAWDWEVDIENGNYDYLMYADIDMDHPEVINELRNWGVWYTNTLNLDGFRIDAVKH
                :.   .   ::*  .*         ::   .: :* * :**        : ***:**.**

Str             IPAQHLEQIRARVGDGS---VYWKQEMIYGAGEAIHPDEYTGAGDVQEFRHAFDIKRIFQ
2DIE_A          IKYSYTRDWLTHVRNTTGKPMFAVAEFWKNDLAAIENYLNKTSWNHSVFDVPLHYNLYNA
                *  .: .:  ::* : :   ::   *:  .   **.    . : : . *  .:. :

Str             NERLAYLDDFGQSWGYLPSAG---AGVFVDNHDTERNGSTLTYRDGSAYTLANVFMLAWN
2DIE_A          SNSGGYFDMRNILNGSVVQKHPIHAVTFVDNHDSQPGEALESFVQSWFKPLAYALILTRE
                .:  .*:*  .   * : .     * .******:: . :  :: :.   .** .::*: :

Str             YGSPDIHSGYEFTDYDAGPPRGGEVRDCYQDNWKCQHAWPEIASMVGFRNAVGDAAVTNW
2DIE_A          QGYPSVFYG----DYYGIPTHG-----------VPSMKSKIDPLLQARQTYAYGTQHDY
                 * *.:. *    ** . *.:*               .:* .::  *:: . .:  ::

Str             WDNGNDVIAFGR-GDRGFVVVNHESAAVDRTWQS-SLPGGTYCDVQSGRAVTVDGSGRFT
2DIE_A          FDH-HDIIGWTREGDSSHPNSGLATIMSDGPGGNKWMYVGKHKAGQVWRDITGNRSGTVT
                :*: :*:*.: * ** ..   .  :   * .  .  :  *.:   *  * :* : ** .*

Str             ASVAAGTALALHAGARDCSGNGGP
2DIE_A          INADGWGNFTVNGGAVSVWVKQ--
                 .. .   ::::.** .   :
```

Figure 5.18: Alignment of target sequence with template 2DIE

As Amy Strplo 716 hydrolyses starch to produce maltotetraose as one of the products, use of the 3D structure of the maltotetraose-forming exo-amylase from *Pseudomonas stutzeri,* (PDB Id: 2AMG) as a possible starting template was also investigated. This structure was determined at a resolution of 2 A^0, and had a very low sequence homology (17% identity) with the target sequence (Fig. 5.19).

Figure 5.19. Alignment of target sequence with template 2AMG

All the homology modeling was done using Prime 3.0 suite program from Schrodinger, 2011 (Schrodinger, 2011). Structure prediction utility of Prime suite was used to align the target sequence with the template structure, for building the model. Once the target and templates were aligned, the next step of model building was to transfer the backbone atom coordinates for the aligned regions and side chains of conserved regions. This was followed by side chain optimization, minimization of non-template residues, building of insertion and closing of deletions in the alignment. The three models are shown in Figs 5.20 to 5.22.

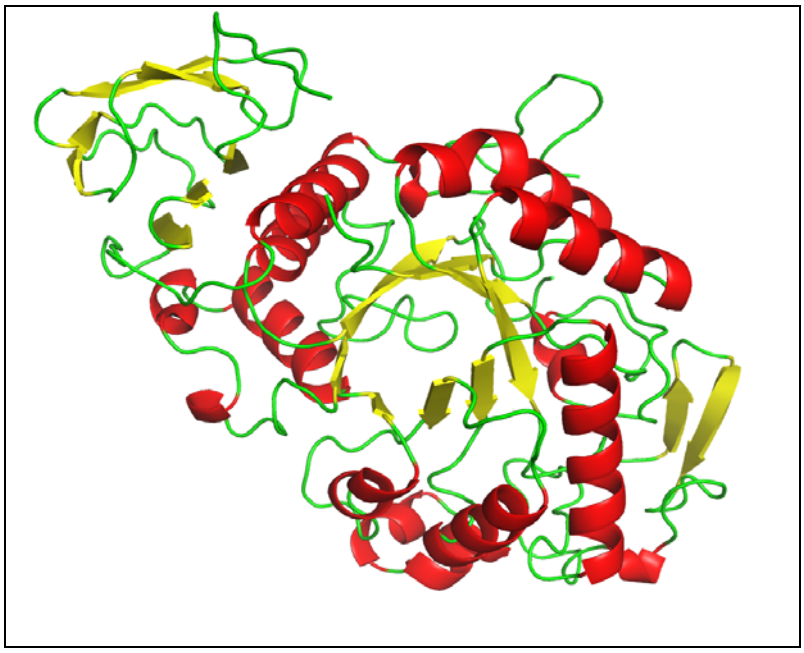


Figure 5.20. Model built with 1B0I

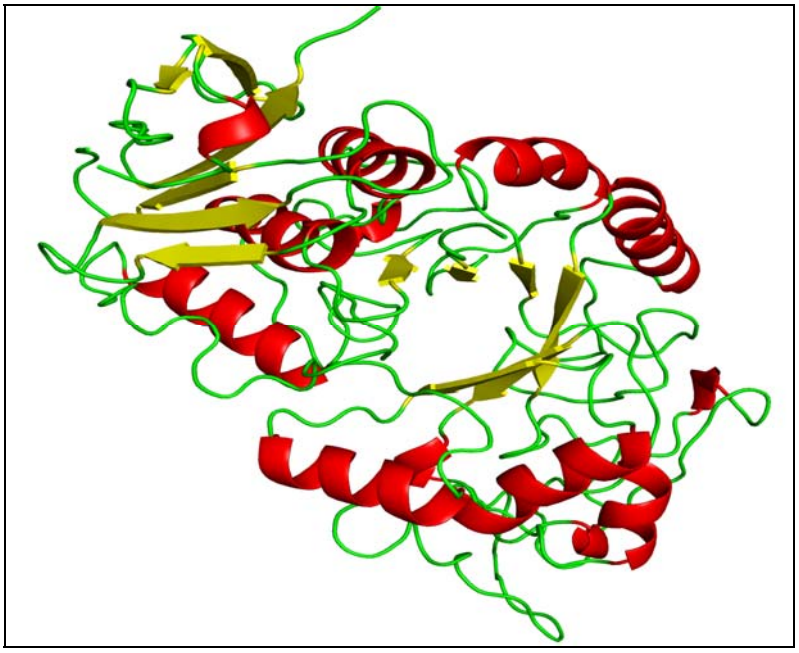


Figure 5.21. Model built with 2DIE

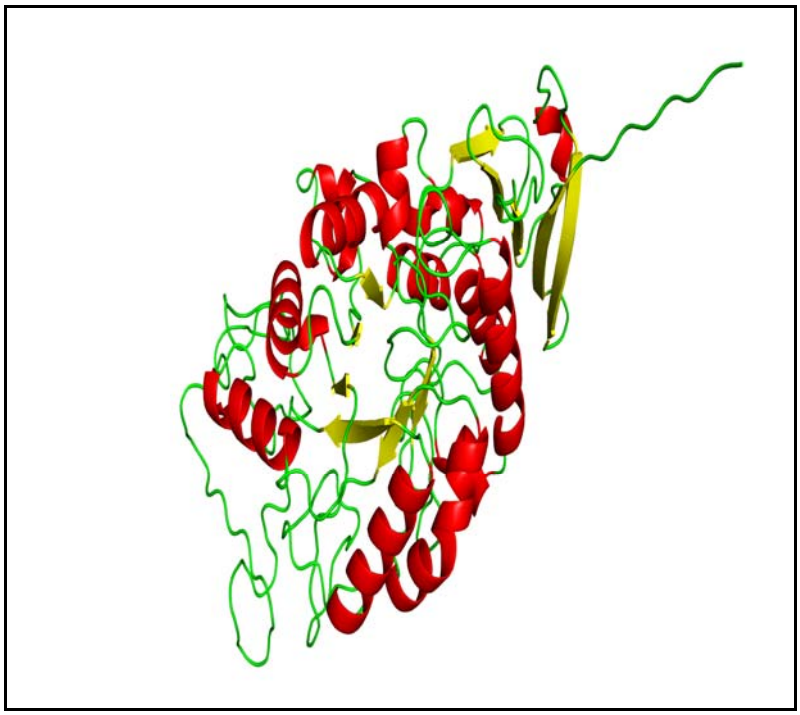


Figure 5.22. Model built with 2AMG

The models were further evaluated for their correct geometry and stereo chemistry by using programs such as verify3D (Eisenberg *et al*, 1997), Errat (Colovos and Yeates, 1993) and PROCHECK (Laskowsi *et al*, 1993). PROCHECK checks the overall quality of model by plotting Ramachandran map, which is a two-dimensional scatter plot between the two dihedral angles (Phi an d Psi) of residues in a polypeptide chain. The overall percentage of residues that are lying in the allowed and disallowed region of this plot is used to assess the overall quality of model. A good model is one having less percentage of residues lying in the disallowed region of plot, hence with less steric clashes. Similarly, Errat is a protein structure validation algorithm, which works by analyzing the statistics of non bonded interactions between different atom types. For any protein structure, Errat gives an overall quality factor, which is used to assess the quality of model. This quality factor is expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. Good high resolution structures generally produce values around 95% or higher. Higher the quality factor, higher is the quality of the model. Verify3D gives quality score to each amino

acid residue of protein. A bad quality residue provides a score less than zero. The overall score of each amino acid residue of a protein helps to assess its quality.

The summary of comparison of the three models is provided in Table 5.9.

Program		**Model with 1B0I**	**Model with 2DIE**	**Model with 2AMG**
	%Identity	48	21	17
Errat	Overall quality factor	91.647	79.310	76.291
Verify3D Plot	% of residues with -ve score	0	4.57	3.43
Procheck	% of residues in most favoured regions	83.3	79.5	75.5
	% of residues in additional allowed regions	14.8	16.4	18.6
	% of residues in generously allowed regions	1.6	3.0	4.4
	% of residues in disallowed regions	0.3	1.1	1.4
	Number of disulfide bonds	2	0	0

Table 5.9. Comparison of the three models based on Errat, Verify3D Plot and Procheck scores.

Among the three models, the model built with template 1B0I is the best model because of its high sequence homology with the template. The overall Errat quality factor for this model is also good (91.647, Figure 5.23). Only 0.3% residues are in the disallowed region of Ramachandran plot (Figure 5.24). Similarly all residues of the model have positive verify3D score (Fig. 5.25) which itself explains that the model is good. This model has disulfide bonds. Similarly Cys140 and Cys121 are also nearer to each other which are present in loop region. So this pair could also form a disulfide bond. So we assume that the true structure could have net 3 disulfide bond. The C-terminal region containing Cys 431 residue (CSGNGGP), could not be modeled. This

Cys 431 is nearer to Cys 396, and could form disulfide bond in the template. However in our model, since the C-teminal region is not modeled, their possibility of forming disulfide bond cannot be ascertained.

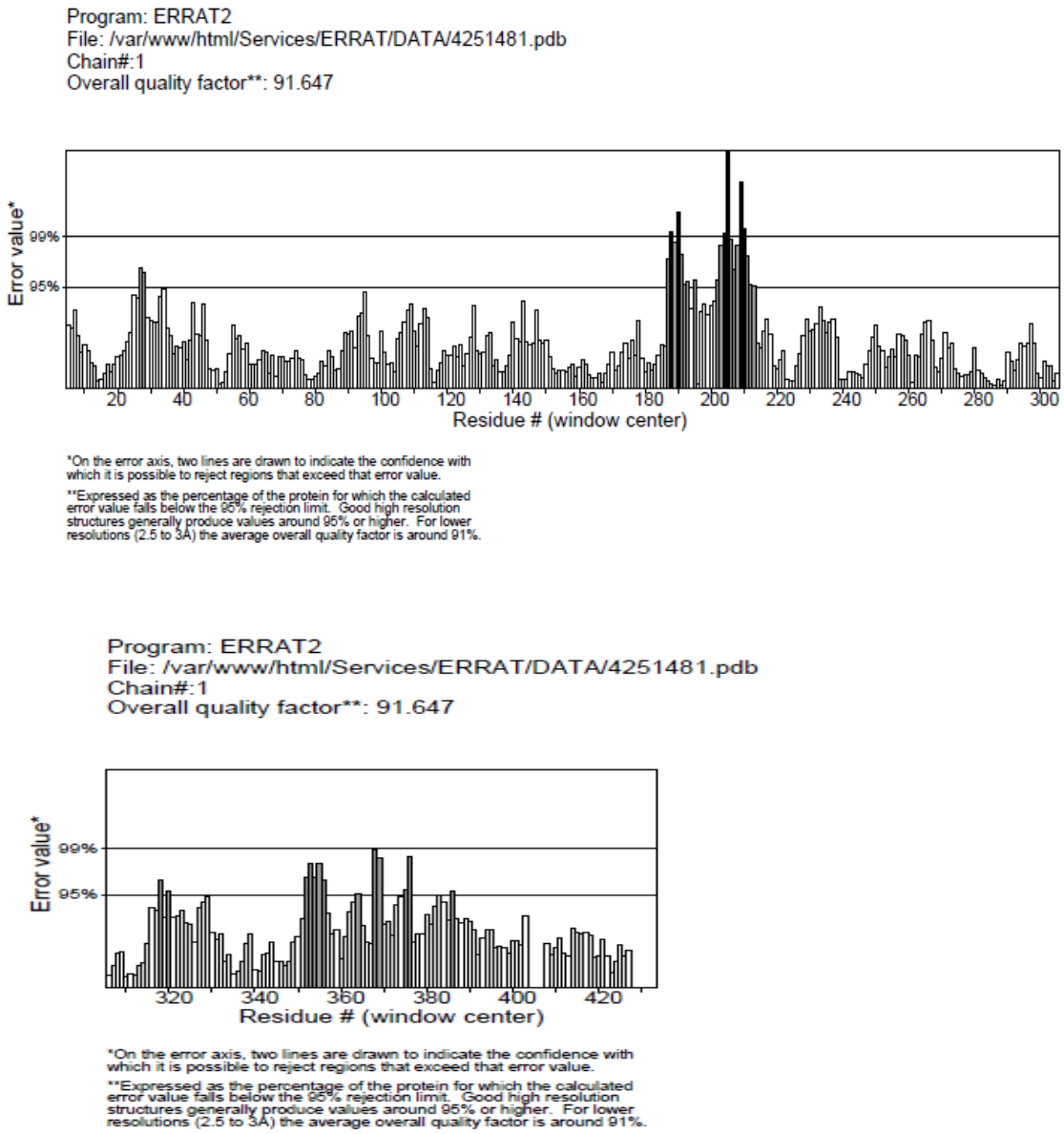


Figure 5.23. Errat plot for the model built with 1B0I as template

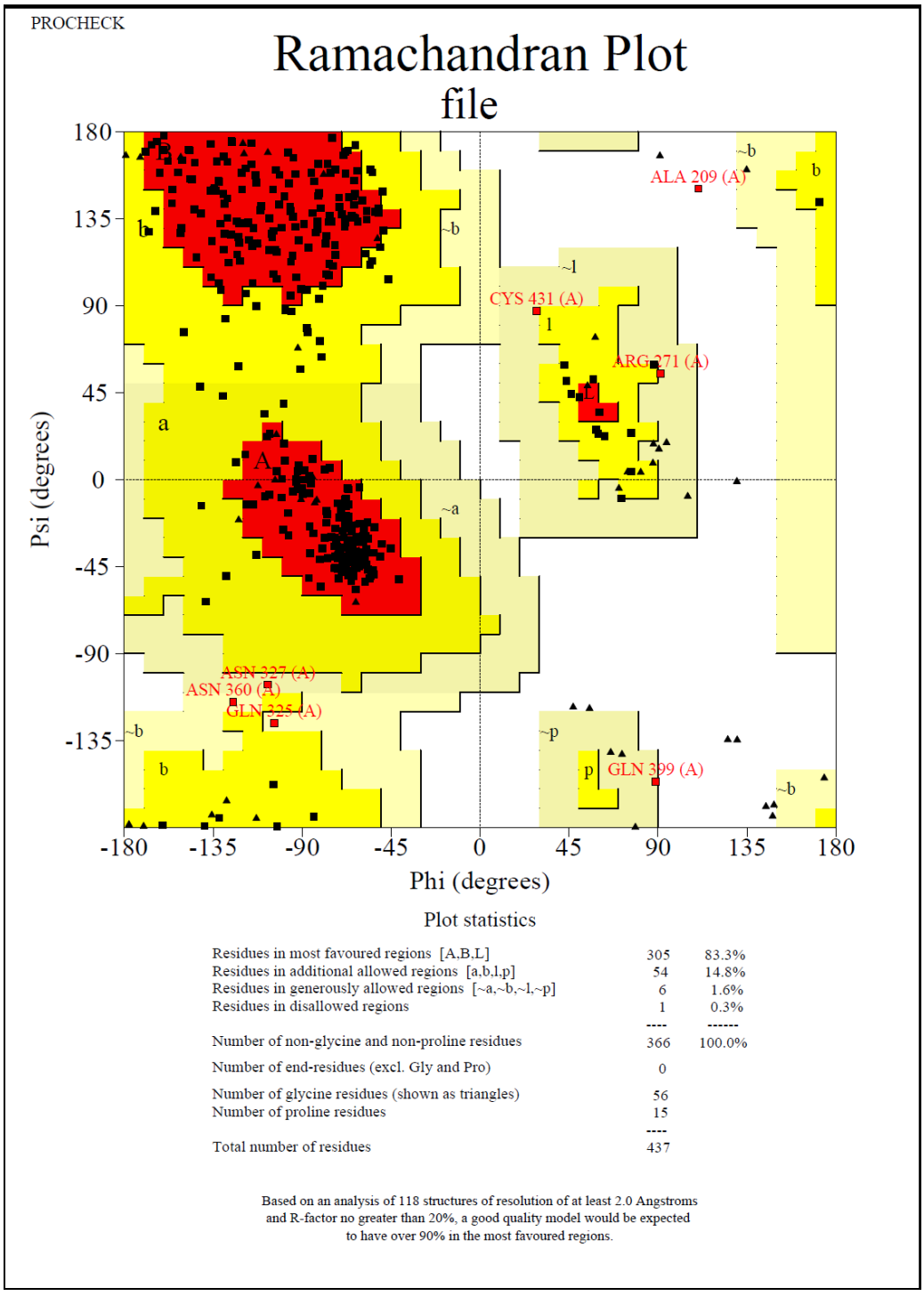


Figure 5.24. Ramachandran plot for the model built with 1B0I as template

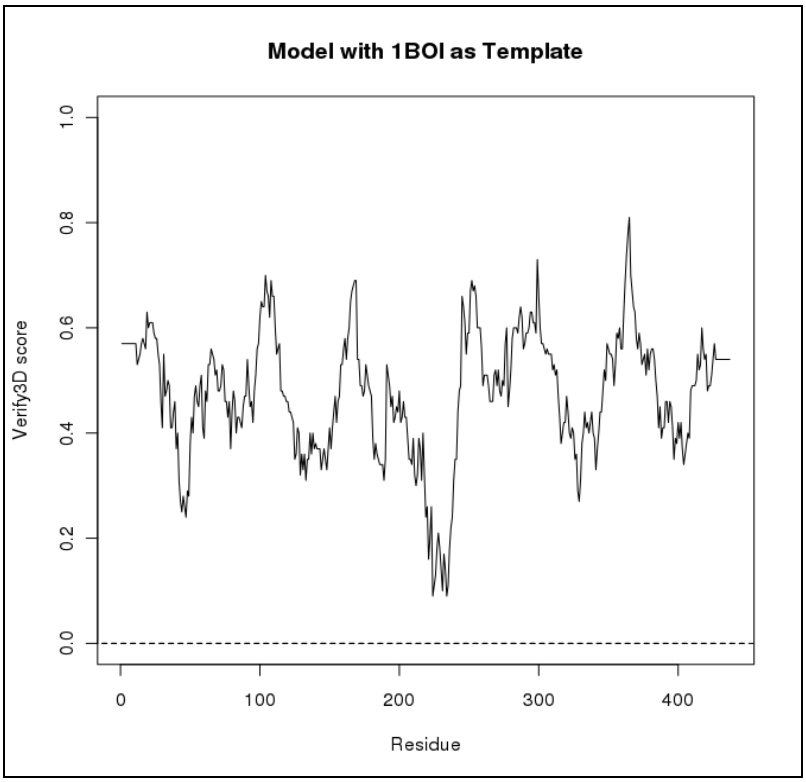
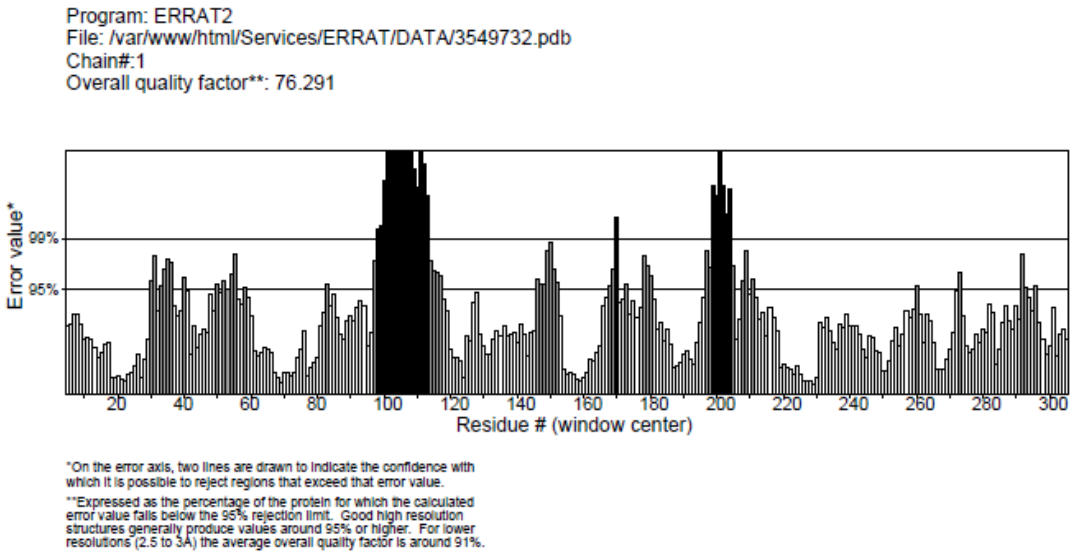


Fig. 5.25. Verify3D plot for the model built with 1B0I as template

The model built with template 2AMG is based on a very low sequence homology between target and template sequences (17%). Overall quality factor obtained from Errat is approximately 76% (Fig. 5.26). 75.5% residues are in the most favoured regions of Ramachandran plot (Fig. 5.27). 3.43% of residues have -ve Verify3D score (Fig. 5.28). It is also observed that the model does not have any disulfide bond.



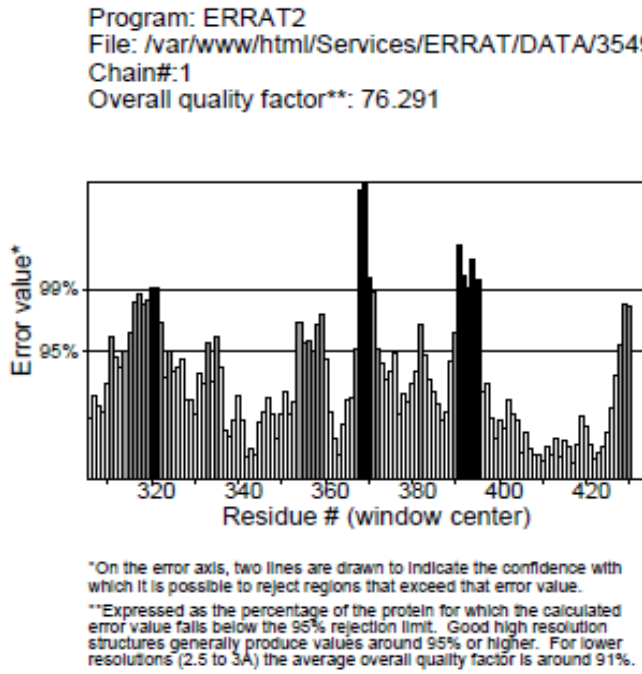


Fig. 5.26. Errat plot for the model built with 2AMG as template

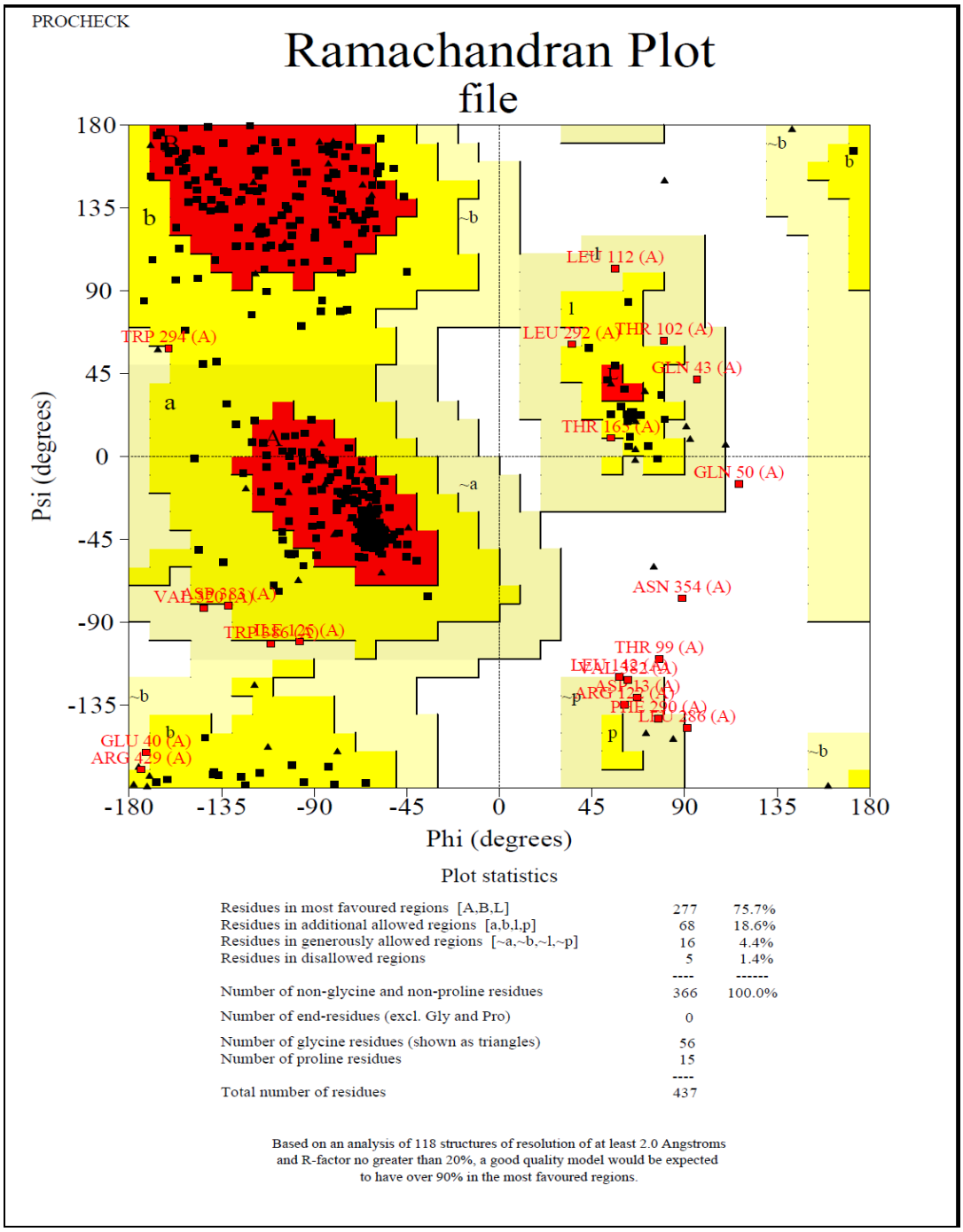


Fig. 5.27. Ramachandran plot for the model built with 2AMG as template

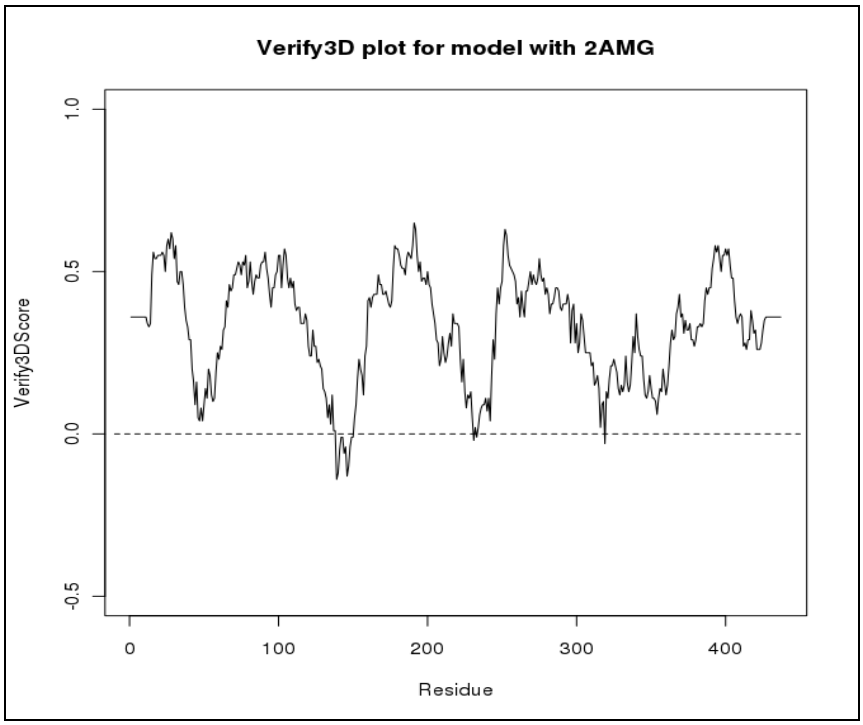


Fig. 5.28. Verify3D plot for the model built with 2AMG as template

The model built with the template 2DIE, is also based on a low sequence homology between the target and template sequences (21%). Overall Errat quality factor is approximately 79.310 (Fig. 5.29). 1.1% of residues are in the disallowed region of Ramachandran plot (Fig. 5.30). 4.57% of residues have -ve Verify3D score (Fig. 5.31). In this model also no disulfide bond is observed.

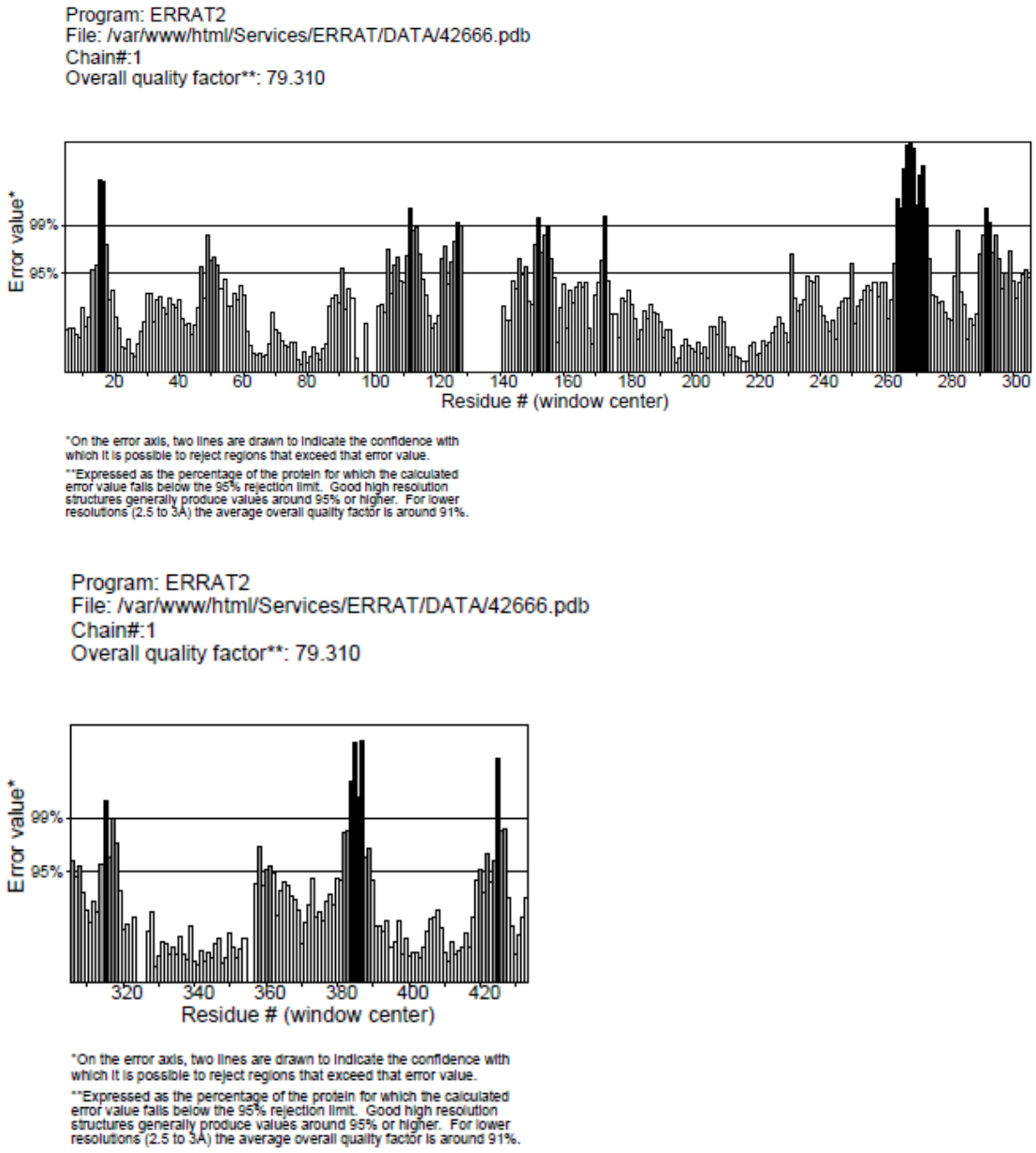


Fig. 5.29. Errat plot for the model built with 2DIE as template

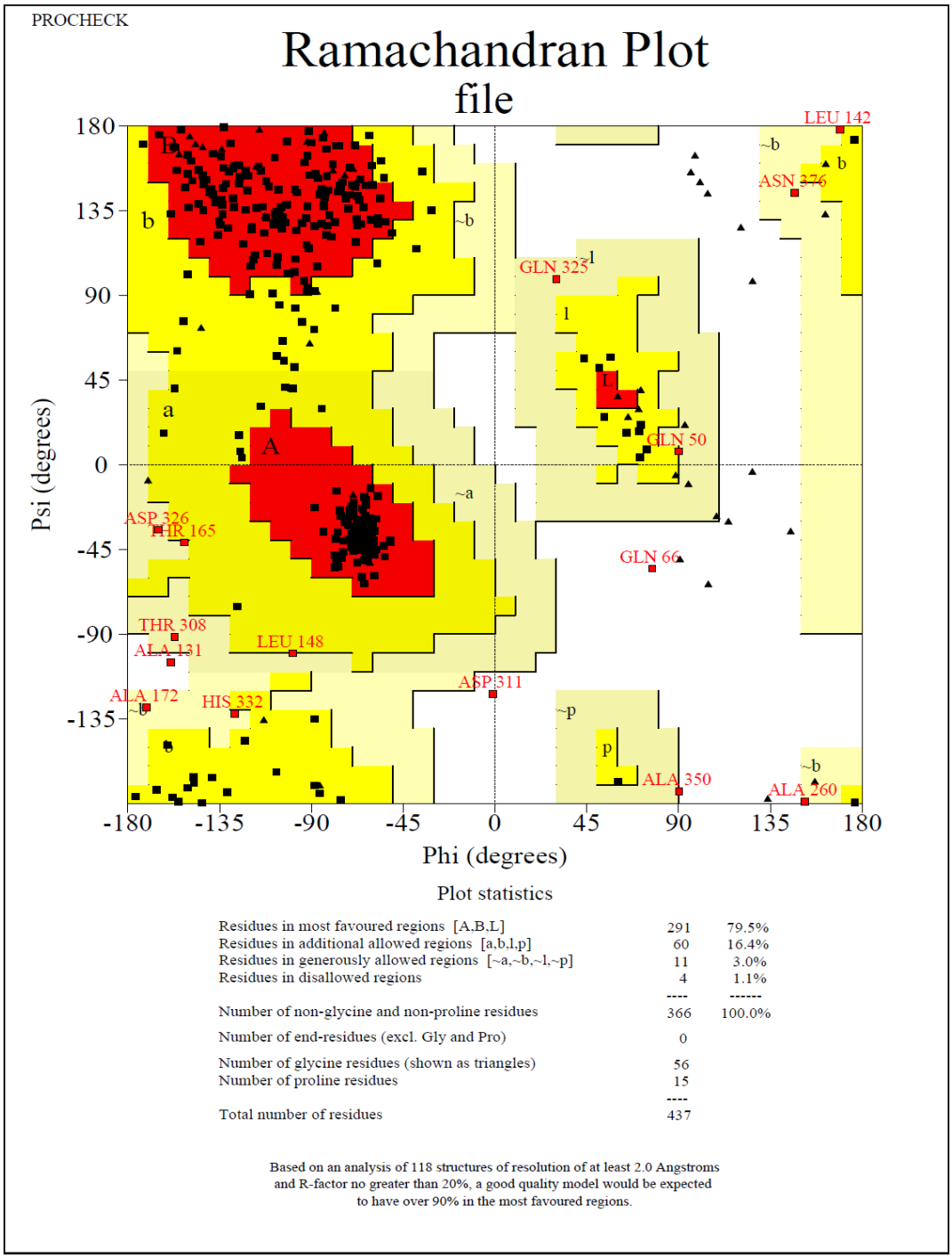


Fig. 5.30. Ramachandran plot for the model built with 2DIE as template

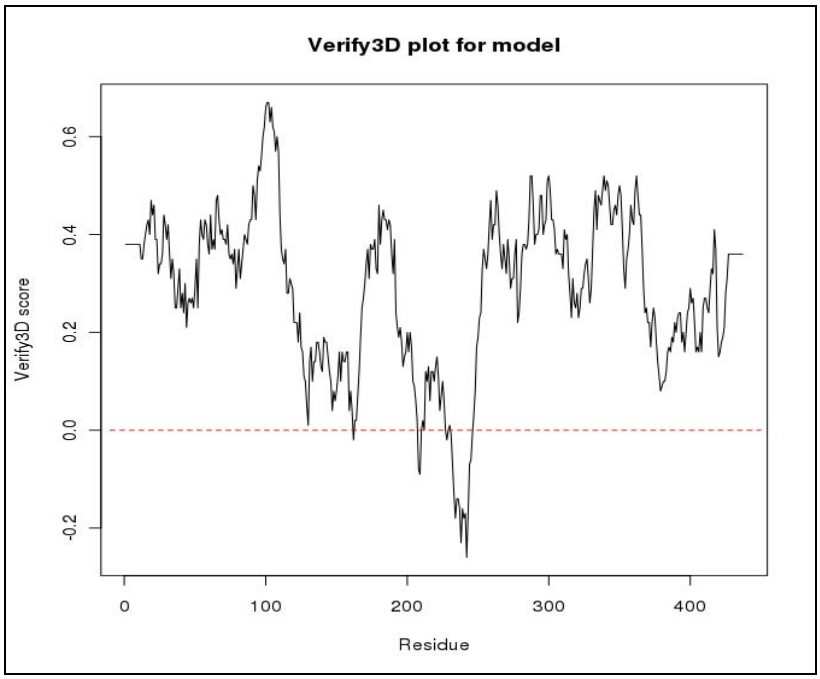


Fig. 5.31. Verify3D plot for the model built with 2DIE as template

From the above analysis, it can be concluded that the model built from 1B0I is the best model. As per PDBsum analysis (Laskowski, 2001) this model has a three domain structure. The domain architecture of this model is shown in Fig. 5.20.

When we compare amino acid composition between the template 2DIE entry of PDB and the target sequence, it is found that the target sequence has a decrease in lysine content followed by an increase in arginine content. Similarly a decrease in asparagines content is counter balanced by an increase in glutamine content. The probable reason might be that "Asn has higher rate of undergoing deamidation than Gln". This trend of decrease of Asn content is a most striking observation in many thermostable proteins.

Conclusions

1) A protocol for the isolation of good quality DNA was optimised by eliminating the contaminating polysaccharides in the very first step.

2) Restriction enzyme analysis of genomic DNA with *Msp I*, *Hpa II*, *Mbo I* and *Sau3AI* suggests that the DNA is highly GC rich and the `CCGG' and `GATC' sequences are by and large unmethylated.

3) 1312 bp of the gene encoding the alkaline α-amylase from *Streptomyces lonarensis* strain 716 (*Amy Strplo 716*) has been sequenced. The partial gene sequence translated into a protein of 437 amino acids (Amy Strplo 716) and has been submitted to the GenBank with the accession number ADZ45287.

4) Amy Strplo 716 shows all the conserved regions present in other Strepyomyces α-amylases and it forms a separate branch in a major cluster in the phylogenetic tree of *Streptomyces* α-amylases.

5) Many of the residues involved in substrate and Ca^{2+} binding are conserved in Amy Strplo 716.

6) The disulfide bond C70-C115 which links domains A and B in PPA and other mammalian α-amylases is absent in Amy Strplo 716 which is a characteristic feature of the α-amylases of microorganisms, invertebrates and insects.

7) The percentage of hydrophobic residues in Amy Strplo 716 is 34.78% which compares well with values from PPA, HPA, HSA and AHA.

8) The G+C content of Amy Strplo 716 is 70.38% which agrees with the high G+C values of Streptomyces.

9) Majority of the codons in Amy Strplo 716 possess either a G or c in the third position with a preference of C over G, a trend that is coomon in Streptomyces.

10) Homology modeling suggests that Amy Strplo 716 has 48% sequence identity with AHA. The homology model of Amy Strplo 716 built using AHA as a template is ideal as it shows favourable scores for Errat, Verify3D plot and Procheck.

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Chapter 6

Discussion

Discussion

The focus of the work presented in this thesis is the alkaline α-amylase secreted by an alkalophilic actinomycetes strain NCL 716. The present study includes

(i) Characterization of the alkalophilic actinomycetes strain NCL 716.

(ii) Optimization of the fermentation media for maximizing the alkaline α-amylase yield.

(iii) Purification and characterization of the alkaline α-amylase, and

(iv) Molecular cloning and sequence analysis of the partial gene encoding the alkaline α-amylase and homology modeling of the translated protein.

(i) Characterization of the alkalophilic actinomycetes strain NCL strain 716.

This alkalophilic actinomycete designated as strain NCL 716 was isolated from the soil surrounding Lonar lake, a salt water meteorite lake, in Buldhana district in the State of Maharashtra in India. This actinomycetes strain has been characterised using a polyphasic approach i.e rDNA analysis, classical and chemotaxonomical approaches. This strain was identified as a *Streptomyces* strain by rDNA analysis and was given the name *Streptomyces lonarensis* strain NCL 716 as it was isolated from the soil around Lonar lake. The GenBank accession number is FJ 919811. Its cell wall was identified as Type I which is characteristic of the genus *Streptomyces*. The strain was found to be a facultative alkalophile which grew at 28°C – 30°C and pH of 8-12. It was non halophilic but could also grow in presence of upto 6% NaCl. This is the sixth report of an alkalophilic *Streptomyces* strain. This strain differed from the two alkalophilic strains *S. gulbargensis* and *S.sodiiphilus* in having a relatively high percentage of iso C16:0 (46.87%) which could possibly explain why this strain does not grow at pH 7 unlike these other two strains. Some of the other important characteristics of this *Streptomyces lonarensis* strain NCL 716 are the white colour of its aerial mycelium, smooth surface of its spores, a Rectus-Flexibilis morphology of its sporophore and production of pigment on ISP 7 medium. It utilized majority of carbon sources except *meso*-inositol, sucrose, lactose. The cell free extract of the strain exhibited

antimicrobial activity against *B.subtilis*, *E.coli*, *S.aureus*, *P.aeuruginosa*, *P.fluorescens and F.proliferatum*. It was resistant to antibiotics Ampicillin, Chloramphenicol, Gentamycin, Novobiocin, Rifampicin and was most sensitive towards Rifampicin (10 μg).

One of the most important characteristics of this strain is its ability to secrete an alkaline α-amylase which has formed the central theme of this thesis. This is the fifth report of an alkaline amylase from an alkalophilic *Streptomyces* strain. The α-amylase hydrolyses starch to produce a mixture of maltotriose, maltotetraose and maltose. This is the first report of an alkalophilic *Streptomyces* strain and the second report of a *Streptomyces* strain producing maltotetraose. This is also the fourth report of a maltotriose producing *Streptomyces* strain.

(ii) Optimization of the fermentation media for alkaline α-amylase production.

As a first step towards characterisation of the alkaline α-amylase from the *Streptomyces lonarensis* strain NCL 716, a cost-effective medium for maximizing the yield of the amylase, was optimised using a combination of Plackett-Burman, SVR and MJWA. A SVR-based process model was developed for approximating the non-linear relationship between the fermentation operating variables and the α-amylase yield. The maximum amylase activity predicted by SVR was in good agreement with the experimental values at the optimized levels. The most influential factor was found to be starch while the least influential factor was peptone. Peptone which is one of the expensive media components was used at a concentration of 1g/L, which made the optimum media composition cost effective. The optimum media composition thus obtained by this method was: Yeast Extract: 4.53g/L, Starch: 20.246g/L, K_2HPO_4: 0.0827%, $MgSO_4$: 0.15%, Peptone: 1g/L. A maximum enzyme activity of 297 U/ml which was achieved using the above approaches compares well with the activity of reported amylases producing maltooligosaccharides.

(iii) Purification and characterization of the alkaline α-amylase

The alkaline α-amylase from *Streptomyces lonarensis* strain NCL 716 was purified by a combination of ammonium sulphate precipitation (90% saturation)

followed by preparative PAGE (5%). The α-amylase from this strain designated as Amy Strplo 716 exhibited an optimum pH and temperature of 9.0 and 45°C respectively. The enzyme was fairly stable in the pH range 7.0 to 9.0 and lost activity beyond this range. At pH 6.0, 70% residual activity was observed while only 22% of the activity was retained at pH 4.0. At the optimum pH of 9.0, the enzyme was stable at 45°C for more than 2 h but rapidly lost activity above 45°C. The relative activity decreased to 58% at 50°C, 15% at 55°C and 5% at 60°C. The molecular weight of Amy Strplo 716 was 37 kDa. The pH-activity profile obtained revealed the participation of two ionizable groups with pKa values of 7.7 and 8.7. The activation energy for this enzyme was estimated to be 18.36 KJ mol^{-1}.

Drastic inactivation of the amylase activity was observed in presence of Hg^{2+} (17% residual activity) which has been reported for other amylases. Increase in the residual activity up to 129 % in presence of Mg^{2+}, 125% in presence of Co^{2+} and 115% in presence of Ca^{2+} was observed. This could be due to the stabilization of active site geometry by these ions. The enzyme lost 50 % of activity in presence of 2 mM EDTA. Almost 85% activity was regained on addition of 1 mM $CaCl_2$ to the EDTA treated enzyme, indicating that Ca^{2+} was required for enzyme activity. Hence, it can be proposed that the enzyme already has Ca^{2+} bound to it.

It was found that one Trp residue when modified was responsible for the inactivation. Substrate production studies also revealed that Trp, His and Cys are probably involved in the active site of Amy Strplo 716. The inactivation of Amy Strplo 716 in lower concentrations of Gdn-HCl suggested the possible involvement of carboxylate groups in the active site of Amy Strp 716. Two acidic acid residues (Asp or Glu) in all α-amylases are essentially involved in catalysis. Time dependence studies did not yield the desired trend due to sudden drop of activity, which suggested that these residues were present in the active site but in a highly reactive form. A slight lower K_m value with amylose substrate as compared with starch and amylopectin suggested that that Amy Strplo 716 had a greater affinity for amylose.

HPLC analyses of the hydrolysis products of starch at 12 h showed production of G4, G3 and G2. These preliminary results suggest a potential application of this enzyme in the food industry for the production of G3/G4 syrup comprising a mixture of

the above three sugars. The reaction mechanism of the enzymes producing oligosaccharides is distinct from that of the usual α-amylases. These enzymes are presumed to have four, five or six subsites from the active centre to produce the particular oligosaccharides and to prevent them from further degradation.

Effect of different chemical and physical denaturants such as guanidine hydrochloride and change in pH on Amy Strplo 716 conformation and activity was monitored by fluorescence spectroscopy and circular dichroism. The enzyme lost the biological activity first and then the overall folded conformation and secondary structure. The intrinsic fluorescence of the protein was monitored at different pH. The fluorescence emission maximum of the protein with the excitation 295 nm was at 357 nm indicating tryptophans to be present in the polar environment.

The chemical modification studies yielded 5 tryptophan residues which are present on the surface. The native Amy Strplo 716 showed fluorescence spectrum with λ_{max} at 357 nm using excitation wavelength of 295 nm indicated that Trp residues were present in the polar environment or were exposed to the solvent. The fluorescence of proteins at 295 nm originates mainly due to tryptophan.

The Stern–Volmer plots obtained for quenching of the fluorescence with iodide ions under native conditions was linear giving K_{sv} value as and 3.9 M^{-1}. As the charged quenchers can access only surface Trp, the quenching by iodide ions indicated density of positive charge around the surface Trp residues. Cs^{+} did not show any effect on the fluorescence of the native or denatured protein. This may be due to the presence of positively charged residues in the vicinity of some of the exposed (or partially exposed) tryptophan residues.

For the denatured protein (6 M GdnHCl treated), the Stern–Volmer plot still retained the upward curvature which indicated collisional and static mechanisms of quenching of the fluorescence even after the change in conformation. The 6 M GdnHCl treated protein sample showed linear Stern–Volmer plots and the K_{sv} value had substantially increased to 19.24 M^{-1} for iodide ions. This could be due to the increased positive charge density around tryptophans due to change in conformation. These results indicated that tryptophans in Amy Strplo 716 were present in different

environments; most of the residues were present on the surface fully exposed to solvent while only some were buried inside hydrophobic environment.

Far UV CD-spectrum at pH 7.2 showed a trough with minima at 208 nm and 222 nm which indicated the presence of both α and β structures in the enzyme. The secondary structure elements determined using the software CD Pro (continell) were 12% (Helix), 33.6% (β – sheet), 21.6% (turns) and 32.4% (unordered). Decomposition analysis of the tryptophan fluorescence spectra of the native Amy Strp 716 by PFAST (http://pfast.phys.uri.edu/pfast/) indicated that the tryptophan present in the native amylase belonged to class III group and were in polar environment.

(iv) Molecular cloning, sequence analysis and homology modeling of the partial gene encoding the alkaline α-amylase.

The partial gene of 1312 bp encoding the alkaline α-amylase from *Streptomyces lonarensis* strain 716 was cloned and sequenced. It is designated as *Amy Strplo 716.* It translated into a protein of 437 amino acids (Amy Strplo 716) and has been submitted to the GenBank with the accession number ADZ45287.

Amy Strplo 716 shows all the conserved regions present in other *Streptomyces* α-amylases. Many of the residues involved in substrate and Ca^{2+} binding are conserved in Amy Strplo 716. The disulfide bond C70-C115 which links domains A and B in PPA and other mammalian α-amylases is absent in Amy Strplo 716 which is a characteristic feature of the α-amylases of microorganisms, invertebrates and insects. The percentage of hydrophobic residues in Amy Strplo 716 is 34.78% which compares well with values from PPA, HPA, HSA and AHA. Such hydrophobic residues have been implicated in increasing the thermal stability of proteins.

The G+C content of Amy Strplo 716 is 70.38% which agrees with the high G+C values observed in *Streptomyces*. Majority of the codons in Amy Strplo 716 possess either a G or C in the third position with a general preference of C over G, a trend that is common in *Streptomyces*.

Homology modeling suggests that Amy Strplo 716 has 48% sequence identity with AHA. The homology model of Amy Strplo 716 built using AHA as a template is ideal as it shows favourable scores for Errat, Verify3D plot and Procheck.

Conclusion : The alkaline α-amylase from the *Streptomyces lonarensis* strain NCL 716 possesses two important properties ie (i) it has a pH and temperature optimum of 9.0 and 45°C and (ii) it hydrolyses starch to produce a mixture of maltose, maltotriose and maltotetraose. This property suggests its potential application in the food industry for the production of maltooligosaccharides which are known to be beneficial for health. Preliminary studies in this laboratory have also shown that this enzyme when used as an additive with a detergent, improves the efficiency of removing starchy stains. Considering the potential application of this enzyme, the present work has included all the studies ie a detailed enzyme characterization and cloning of the gene encoding the α-amylase, which are required before a recombinant DNA technology can be developed for this enzyme.