STUDIES ON PULLULAN-HYDROLYSING ACTIVITY FROM Sclerotium rolfsii

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BY

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CONTENTS

DECLARATION

Certified that the work incorporated in the thesis "Studies On the Pullulan-Hydrolysing Activity From *Sclerotium rolfsii*" submitted by Mr. H.S. Kelkar was carried out by him under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

M.V. Deshpande

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(Research Guide)

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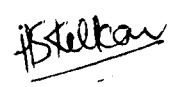

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LIST OF ABBREVIATIONS

DNS	Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
gm	Gram
h	Hour(s)
mg	Milligram
min	Minute(s)
Tris	Tris(hydroxylmethyl)aminomethane
μg	Microgram
μl	Microliter
μmole	Micromole
SDS	Sodium dodecyl sulphate
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Micro-organisms are known to produce a variety of extracellular polysaccharides having unusual properties. Some of these have excellent solubility, adhesive nature, viscosity, plasticity, spinnability etc. Bio-degradability is also an important property, which assures easy recycling of these valuable resources. Xanthan gum produced by *Xanthomonas campestris* , dextran from *Leuconostoc mesenteroides* and pullulan elaborated by *Aureobasidium pullulans* are prominent in the list of industrially important microbial exo-polysaccharides.

Pullulan, a water-soluble exo-polysaccharide, is a polymer of repeating maltotriose (three α-1,4-linked glucose residues) units that are polymerized via α-1,6-linkages. Conventionally, pullulan has been used as a model polysaccharide for the starch-debranching enzyme, "pullulanase" (pullulan 6-glucanohydrolase EC 3.2.1.41) which acts on the α-1,6-linkages in pullulan liberating maltotriose. The amylopectin and glycogen debranching ability of pullulanase has proved to be of considerable importance in the structural elucidation of these and other polysaccharides. Pullulanase is used in saccharification of starch, either to glucose (in combination with amyloglucosidase) or to maltose (in combination with β-amylase) to obtain high yields of the desired products in a short period of time.

There are very few reports of enzymes of fungal origin which act on pullulan. We have identified *Sclerotium rolfsii* (NCIM 1084), as a potential source of pullulan-hydrolysing activity. The present thesis describes investigations on the optimization of pullulan-hydrolysing enzyme production, and its purification and characterization. The study also includes its potential application in starch hydrolysis using protoplast technology.

The work is presented in five chapters and a brief outline of the studies carried out is as follows.

Chapter I: Introduction

This part of the thesis describes a review of the literature on the production and properties of pullulan. The biochemistry of microbial pullulan-degradation has been discussed extensively along with the technological applications of the pullulan-hydrolysing enzymes.

Chapter II: Materials and methods

In this chapter the experimental and analytical methods used for the study have been elucidated.

Chapter III: Optimization of pullulan-hydrolysing activity production in S. rolfsii

Studies on the effect of various inorganic nitrogen and carbon sources, surfactants and organic supplements on the production of pullulan-hydrolysing activity in shake-flask have been discussed. In an optimized medium (1% soluble starch as a carbon source), supplemented with corn steep liquor (1% w/v), maximum levels of the enzyme activity (1.8 IU/ml) were obtained. The enzyme activity was found to be produced constitutively.

In enzymatic hydrolysis of carbohydrates, there is a need to use elevated temperatures to achieve optimal reaction rates. However, increased temperatures can at the same time bring about thermal denaturation of the enzyme protein with eventual loss of catalytic activity. Sugar alcohols, sugars and inorganic salts are known to provide a stabilizing effect against thermal denaturation to various enzymes. The role of some of the stabilizing agents in improvement of the thermal

stability of pullulan-hydrolysing activity was adjudged. With sorbitol (1.5 M), the pullulan-hydrolysing activity remained stable at 60°C, with no apparent loss of activity even after 7 h.

Chapter IV: Purification and characterization of pullulan-hydrolysing activity from S. rolfsii

The enzyme acting on pullulan was purified to homogeneity from the culture filtrate of a ten-days old culture of *S. rolfsii*. The methods included concentration of the culture filtrate by ultrafiltration (Amicon PM-10 membrane) followed by ion exchange chromatography on DEAE-Cellulose DE-52 and gel filtration on Bio-Gel P-150. The protein migrated as a single band in polyacrylamide disc gel electrophoreses carried out at pH 2.9 and 7.5. It appeared to be composed of a single polypeptide chain on SDS-PAGE and the molecular weight of the enzyme was estimated to be 64,000 daltons. The enzyme was found to act both on pullulan and starch. The optimum temperature for hydrolysis of pullulan was 50°C, whereas for starch it was 65°C. The optimum pH (4.0 - 4.5, 0.1 M acetate buffer) for activity and stability was similar for both of the substrates.

No significant effect on of the activity on pullulan, as well as starch, was observed with Mg^{++}, Co^{++}, Ca^{++}, Li^{+}, Mo^{++} and Mn^{++} at a level of 0.005 M in 0.05 M acetate buffer at pH 4.9. Inhibition of the enzyme activities to the extent of 30-70% was observed with Fe^{+++}, Hg^{++} and Pb^{++}. p-Hydroxymercury benzoic acid (1 mM) inhibited the enzyme activities completely.

Purified pullulan-hydrolysing activity was able to hydrolyze a variety of oligo- and poly-saccharides having α-1,4- and α-1,6-linkages (with release of glucose). Some of these were pullulan, starch, amylose, amylopectin, various

malto-oligosaccharides, and dextran. Based on the data obtained with studies on the initial rates of hydrolysis of different substrates and the production of glucose as the sole product of hydrolysis, the enzyme was characterized as a 1,4-α-D-glucan glucohydrolase (EC 3.2.1.3), which showed a significant hydrolysis of pullulan. The enzyme in the present study appeared to be very similar to the glucoamylase "S" described in case of *Cladosporium resinae*. (McCleary and Anderson, 1980, *Carbohydr. Res.*, **86**, 77-96).

Chapter V: Rapid isolation of S. rolfsii protoplasts and their potential application for starch hydrolysis

Investigations on the extracellular enzyme secretion by filamentous fungi are hampered by the fact that the enzymes remain bound to or within the cell wall for some time. In case of *S. rolfsii*, the mycelial clump formation during normal growth further hampers its use. To overcome some of these limitations, studies were undertaken to optimize conditions for rapid isolation and regeneration of *S. rolfsii* protoplasts, and on their subsequent application in enzyme secretion studies.

Optimum yields of protoplasts were obtained in 4 h when 50 mg of mycelium (24 h-old) was incubated with 5 mg of NovoZym 234 in one ml of maleic acid-NaOH buffer (0.05 M, pH 5.0) stabilized with 0.6 M KCl. For the regeneration of *S. rolfsii* protoplasts, sucrose (0.6 M) was found to be the best stabilizer (regeneration frequency ~ 70%).

Since *S. rolfsii* is known to produce amylolytic activities, the potential of the protoplasts of *S. rolfsii* (derived from 48 h old mycelium) for secretion of these enzymes and the hydrolysis of starch was investigated. The protoplasts were stabilized by entrapment in calcium alginate (2% w/v) gel beads and were tested for release

of amylolytic enzyme activities. Maximum levels of α-amylase, glucoamylase and pullulan-hydrolysing activities were obtained in case of free and immobilized protoplasts at 48 and 72 h, respectively, at 30°C. The entrapped protoplasts were able to hydrolyze tapioca and potato starches (1%) with a conversion efficiency of 55% and 44%, when compared with free protoplasts (in terms of glucose produced at the end of 96 h). In batch operations, the immobilized preparation retained 57% of initial activity after 3 cycles (48 h) at 30°C and pH 5.0.

CHAPTER I

INTRODUCTION

CHAPTER 1

1.1 INTRODUCTION

Polysaccharides from natural sources are an important industrial resource that finds extensive application as rheology modifiers (thickening, stiffening and setting agents), in a variety of commercial applications (Margaritis and Pace, 1985; Yalpani and Sandford, 1987). It is during the last decade or two that polysaccharides from microbial origins have been successfully explored and applied in commercial practice (Lawson and Sutherland, 1978; Margaritis and Pace, 1985).

Microbial exopolysaccharides are known to contain a variety of monosaccharide repeating units, which include hexoses, methyl pentoses, oxo-sugars, uronic acids and amino sugars (Table 1.1) (Lawson and Sutherland, 1978). Inorganic ions such as Na^+ and K^+ are also found to be associated with certain polysaccharides like xanthan gum (Margaritis and Pace, 1985). The biosynthesis of the exopolysaccharides, in general, is known to occur most favorably under growth conditions of nitrogen limitation i.e. high C:N ratio. In addition, the synthesis has also been demonstrated to occur in cells in the stationary phase of growth (Slodki and Cadmus, 1978). However, with the exception of xanthan gum and bacterial alginates, the biochemical pathways leading to the polysaccharide synthesis have remained unelucidated (Pace and Righelato, 1980; Betlach *et al.* 1987).

* Same as in P. chrysogenum. Maybe the sugars cannot be metabolized due to lack of N and are therefore unmetabolized.

Table 1.1 : Industrially important microbial exo-polysaccharides

Name	Organism	Sugar Monomer
1. Dextran	*Leuconostoc mesenteroides*	Glucose
2. Xanthan gum	*Xanthomonas campestris*	Glucose Glucuronic acid Mannose
3. Pullulan	*Aureobasidium pullulans*	Glucose
4. Scleroglucan	*Sclerotium glucanicum*	Glucose
5. Microbial alginate	*Azotobacter vinelandii*	Mannuronic acid Glucuronic acid
6. Curdlan	*Agrobacterium* sp. *Alcaligenes faecalis*	Glucose Glucose

1.2 PULLULAN

Bauer (1938) observed the presence of viscosity in cultures of *Pullularia*, a fungal isolate, under conditions of excess carbon in the growth medium. Bernier (1958) suggested that an exopolysaccharide produced was responsible for the viscosity in cultures of *Pullularia*. Bender *et al.* (1959) isolated and characterized the extracellular glucan, which was further termed as "Pullulan". It was subsequently characterized as a polymer of α-1,4-linked maltotriose units polymerized on the terminal glucose residues via α-(1->6)-linkages (Bouveng *et al.*, 1963 a,b; Wallenfels *et al.*, 1965).

1.2.1 Sources of pullulan

Aureobasidium pullulans (De Bary) Arnaud, named as "black yeast", has been used for the production of pullulan (Bouveng *et al.*, 1962). In nature, *A. pullulans* is observed in the phyllosphere as a saprophyte and also on a variety of fruits. It is a polymorphic organism capable of growing either in an yeast form or in a mycelial-form depending on the environmental and nutritional conditions (DeHoog and McGinnis, 1987). Taxonomically, *A. pullulans* is classified under class Ascomycetes, order Dothideales and the sub-order Dothideineae (DeHoog and McGinnis, 1987).

Bouveng *et al.* (1963b) found that *A. pullulans* produces three extracellular glucan components. One was pullulan, the second one an "acidic" β-linked glucan, containing β-(1->3)- and β-(1->6)-linkages which was precipitated by the addition of cetyl trimethyl ammonium hydroxide. An acidic

heteropolysaccharide containing galactose, glucose, mannose and hexuronic acid was detected as the third glucan component. Pullulan was found to be the major fraction of the extracellular glucan components (Bouveng *et al.*, 1963b).

Other than *A. pullulans*, *Tremella mesenterica* NRRL-Y6158 was also reported to produce an extracellular linear glucan composed of 200 α-D-glucopyranose units (Fraser and Jennings, 1971). The glucan was found to contain α-(1->4)- and α-(1->6)-glucosidic linkages in the proportion of 2:1 and was characterized as pullulan. *Tremella mesenterica* is a homobasidiomycetous yeast belonging to the order Tremellales and the family Tremellaceae (Bandoni, 1987; Prillinger, 1987).

There were two more reports on the production of polysaccharides resembling pullulan. Waksman *et al.* (1977) reported the presence of an α-(1->4) and α-(1->6) linked glucan in the fruiting bodies of *Cyttaria harioti* FISCHER, an obligate parasite of South American tree species, *Nathofagus*. Structural similarity of the polysaccharide to pullulan was confirmed by its enzymatic hydrolysis with pullulanase and glucoamylase. Methylation analysis of the polysaccharide estimated the ratio of α-(1->4): α-(1->6) linkages at around 2.4:1, which was close to the value reported for pullulan (2.2:1) produced by *A. pullulans* (Bouveng *et al.*, 1963b).

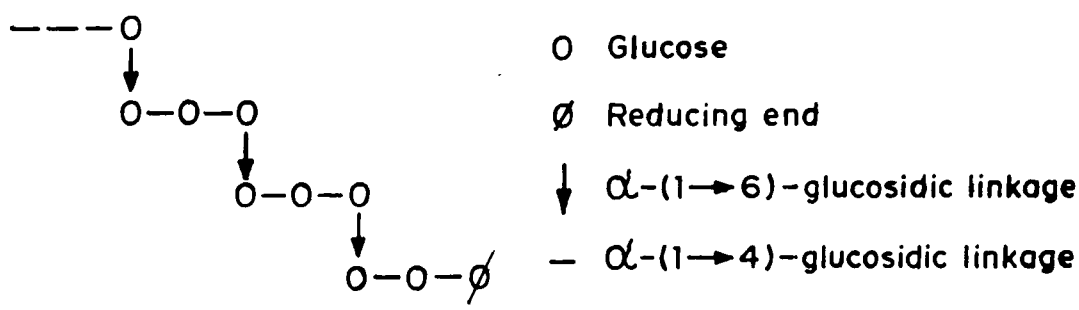
Recently, a taxonomically related species, *Cyttaria darwinii* (Order, Discomycetes; Family, Cyttariaceae) was reported to have pullulan in its stromata (Oliva *et al.*, 1986).

1.2.2 Structure of pullulan

Based on the optical rotation ([α]$_D$ between 180° and 190°) and partial acid hydrolysis studies, Bender *et al.* (1959) proposed a predominance of α -(1->4)- and α -(1->6)-glucosidic linkages in the pullulan. Periodate oxidation revealed that the ratio of α -(1->4): α -(1->6) glucosidic bonds was 2.2:1 (Bouveng *et al.*, 1963b; Wallenfels *et al.*, 1965).

Around the same time, Bender and Wallenfels (1961) isolated an enzyme from *Aerobacter aerogenes*, which was able to hydrolyse pullulan to maltotriose. This enzyme was termed as "pullulanase" (pullulan 6-glucanohydrolase, EC 3.2.1.41).

Using these observations, a structure was proposed for pullulan wherein maltotriose units were polymerized on the terminal glucose residues via α -(1->6) glucosidic linkages. Wallenfels *et al.* (1965) presented a schematic structure of pullulan as follows:



In the enzymatic hydrolysate of pullulan by pullulanase, a minor amount of a tetrasaccharide was observed along with maltotriose (Catley, 1970; Catley and Whelan, 1971). The tetrasaccharide was isolated and identified as maltotetraose

by its hydrolysis with sweet potato β-amylase, which yielded only maltose. Catley and Whelan (1971) suggested a modification in the structure of pullulan which was able to account for the presence of maltotetraose. They suggested that maltotetraose was an integral part of the pullulan chain and was replacing maltotriose-residues in some positions to form α-(1->6)-glucosidic linkages on the terminal glucose residues without affecting the linearity of the pullulan molecule.

Other investigations on the structure of pullulan have led to reports of presence of some α-(1->3)-glucosidic linkages (Sowa *et al.*, 1963). They reported the isolation of crystalline acetate of nigerose (D-Glc-α-(1->3)-D-Glc) from pullulan providing evidence for the presence of α-(1->3)-linked sugars, though these were estimated to be present at a very low concentration (6%) (Sowa *et al.*, 1963).

Molecular weight of pullulan was normally found to be of the order of 10^4 - 10^6 daltons (Ueda *et al.*, 1963, Wallenfels *et al.*, 1965).

1.2.3 Production of pullulan

Considerable attention has been given to the optimization of conditions for the production of pullulan. Traditionally, *A. pullulans* AHU 9553, *Dematium pullulans* IFO 4464 and *Pullularia fermentans* var. *fermentans* IFO 6401 were some of the strains used for the production of pullulan. The desirable characteristics of the strains under study were low pigment production, a faster growth rate and the ability to consistently produce a high molecular weight product.

Yuen (1974) reported that economically viable pullulan production can be carried out using partial hydrolysates of starch as a carbon source in pullulan fermentation. Pullulan is not produced through out the growth period of the organism, but is produced only during the late exponential phase with an excess of carbon source together with a growth limiting nitrogen source (Catley 1971a; 1973). The level of metabolizable nitrogen source present in the medium played an important role in the production of pullulan. A variety of organic nitrogen sources were assimilated by *A. pullulans*. These included corn gluten, soybean, peptone, nitrate and ammonium salts (Catley, 1971b). Catley (1971b) reported a pronounced inhibitory effect of elevated levels of ammonium ions on the production of pullulan, the reason behind which is still unclear. Recently, Deshpande and Rale (1990) have reported the use of cheese whey as a novel carbon source for the production of pullulan. It was also noted by them that the choice of nitrogen source used in the medium affected the production of pullulan and the production of pigment.

A. pullulans is capable of polymorphic growth depending on the environmental conditions. Catley (1973) first suggested that there may be some correlation between the cell morphology of *A. pullulans* and the production of pullulan. It was reported that the synthesis of pullulan appeared to be associated with the yeast-like cells of the organism. It was observed that the onset of production of the exopolysaccharide coincided with the appearence of buds from the hyphae that led to the formation of yeast-like cells.

* see my earlier comment!

1.2.4 Biosynthesis of pullulan

Most of the exo-polysaccharides were assumed to be synthesized by the cell in an analogous mechanism to that found to be involved in the synthesis of cell wall polysaccharides (Margaritis and Pace, 1985). Dextran, appeared to be a notable exception wherein the synthesis of the polysaccharide was catalyzed by dextransucrase in the extracellular environment (Pace and Rhigelato, 1980).

The biosynthetic pathway leading to the production of pullulan is still not completely elucidated. Taguchi *et al.* (1963) reported the involvement of a lipid moiety in the pullulan synthesis. Recently, Catley and McDowell (1982) studied the lipid intermediates formed during the biosynthesis of pullulan. They were able to isolate radioactive glycolipids from cells of *A. pullulans*, pulsed with D-[^{14}C]-glucose. The analysis of the labelled glycolipid fractions indicated that glucose, isomaltose, panose and isopanose linked to lipid by pyrophosphate bonds were synthesized by *A. pullulans*. Since, *A. pullulans* does not contain any α-(1->4)- and α-(1->6)-linked glucosyl residues in its cell wall, these were considered as precursors for the biosynthesis of pullulan. Correlating these results, Catley and McDowell (1982) proposed a scheme for the assembly of these activated oligosaccharides into pullulan chains. However, the biosynthesis of pullulan is still not clear.

1.3 PULLULAN-HYDROLYSING ENZYMES

1.3.1 Occurrence of pullulan-hydrolysing enzymes

Pullulan-hydrolysing enzymes are produced mainly by microorganisms (Saha and Zeikus, 1989). Initially, only the enzyme from *A. aerogenes* (presently called *Klebsiella oxytoca*) has been featured widely in the literature and has been extensively characterized over the last decade (Mercier *et al.*, 1973; Ohba and Ueda, 1975; Brandt *et al.*, 1976). The molecular genetics of synthesis and secretion of pullulanase from *K. oxytoca* has also been extensively investigated by a number of researchers (Michaelis *et al.*, 1985; Chapon and Raibaud, 1985; Pugsley *et al.*, 1986; Kornacker *et al.*, 1989, Pugsley and Reyss, 1990). Other than *K. oxytoca*, the presence of pullulanase has been reported mainly in the genus *Bacillus* (Nakamura *et al.*, 1975, Takasaki, 1976b; Fogarty and Griffin, 1975; Adams and Priest, 1977; Kusano *et al.*, 1988; Castro *et al.*, 1990).

Pullulan has been used as a model polysaccharide for the detection of amylopectin debranching enzymes, which is a constituent polymer of starch. The α-(1->6)-linkages present in pullulan are taken to mimic the α-(1->6)-linked branch points in the amylopectin. Hence, microorganisms capable of producing pullulan-hydrolysing enzymes become potential sources of the starch debranching enzymes. During the decade of 1970s, the successful commercialization of processes for the production of starch-hydrolysing enzymes paved the way for the industrial application of pullulanases (Norman, 1981). Its inclusion in the

enzymatic starch hyrdolysis was shown to enhance the rate of saccharification of starch. It was also found to facilitate complete conversion of the starch to glucose syrup (98 - 99% glucose) (Norman, 1981, Saha and Zeikus, 1989).

1.3.2 Types of pullulan-hydrolysing enzymes

Bender and Wallenfels (1961) were the first to report the presence of an enzyme from *A. aerogenes*, that was capable of hydrolysis of the α-(1->6)-glucosidic linkages in pullulan leading to the production of maltotriose. This enzyme was later on termed as "pullulanase" (pullulan 6-glucanohydrolase, EC 3.2.1.41). The reaction catalyzed by the enzyme was characterized as an endo-hydrolysis of the pullulan and also the α-, β-limit dextrins of amylopectin and glycogen (products of extensive hydrolysis of the polysaccharides by α- or β-amylase, respectively) (Wallenfels *et al.*, 1965, Mercier *et al.*, 1973).

Sakano *et al.* (1971) reported a new class of enzymes capable of hydrolysis of pullulan at one of the two α-(1->4)-glucosidic linkages present in maltotriose, the repeating unit of pullulan. The predominant product of hydrolysis was identified to be isopanose (maltosyl- α-(1->4)-glucose) (Sakano *et al.*, 1972). Subsequently, the enzyme was designated as "isopullulanase" (pullulan 4-glucanohydrolase, EC 3.2.1.57)

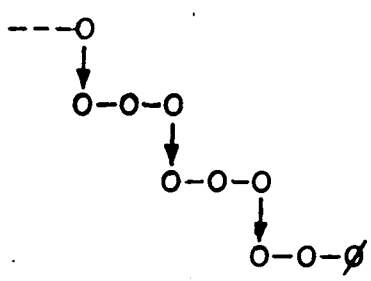
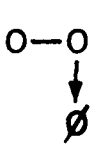
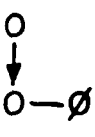
Marshall (1975) reported the presence of a fungal glucoamylase (1,4- α-D-glucan glucohydrolase, EC 3.2.1.3) from *Cladosporium resinae* (now called *Hormoconis resinae*) capable of exo-hydrolysis from the non-reducing end

of pullulan chain, producing glucose as the sole product of hydrolysis. Saha *et al.* (1979) subsequently reported the presence of glucoamylase I in *Aspergillus niger*, which had a similar type of hydrolytic action on pullulan.

"Neopullulanase" (a tentative designation) is one of the two latest classes of enzymes reported to hydrolyze pullulan. Kuriki *et al.* (1988a) isolated and purified neopullulanase from *Bacillus stearothermophilus* strain TRS 40. The enzyme was shown to produce panose (D-glucosyl- α -(1->6)-maltose) as the predominant product of hydrolysis of pullulan (Imanaka and Kuriki, 1989).

During the last two years, a totally new class of enzymes was discovered in thermophilic organisms that was capable of pullulan hydrolysis (Saha and Zeikus, 1989; Antranikian, 1990). These enzymes were shown to hydrolyse pullulan at the α -(1->6)-glucosidic linkages to give maltotriose as the sole product of hydrolysis by a mechanism resembling the pullulanase (EC 3.2.1.41). But in contrast to pullulanase, these enzymes hydrolysed α -(1->4)-glucosidic linkages in amylose. It resulted in the formation of a number of products with a varying degree of polymerization (DP) (DP2 - DP5) by a reaction mechanism analogous to α -amylase (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1). These enzymes, now tentatively designated as "amylopullulanases", represent a group of enzymes having dual hydrolytic specificity depending on the the substrate being used (Saha and Zeikus, 1989).

Information presently available about the various classes of enzymes capable of pullulan hydrolysis can be presented as follows (modified from Saha and Zeikus, 1989):

Substrate	Product	Enzyme
 Pullulan	 Maltotriose	Pullulanase (EC 3·2·1·41)
	Isopanose	Isopullulanase (EC 3·2·1·57)
	 Panose	Neopullulanase (tentative)
	Ø Glucose	Glucoamylase (EC 3·2·1·3)
	O—O—Ø (from pullulan)	Amylopullulanase (tentative)
	O—Ø Maltose O—O—Ø Maltotriose (from starch)	

1.3.3 Purification and general properties of pullulan-hydrolysing enzymes

A variety of chromatographic procedures and fractional precipitaion techniques have been used for the purification of different pullulan-hydrolysing enzymes. Ion exchange chromatography on DEAE-cellulose has been employed in many cases for purification of pullulanase (Odibo and Obi, 1988, Ohba and Ueda, 1973, Ueda and Ohba, 1972, Nakamura *et al.*, 1975). It has also been used for the fractionation of isopullulanase in case of *Aspergillus niger* by Sakano *et al.* (1972). Neopullulanase from *Bacillus stearothermophilus* TRS 40 was successfully fractionated using DEAE-cellulose chromatography by Kuriki *et al.* (1988a). It has also been a method of choice for the isolation a number of amylopullulanases (Plant *et al.*, 1986; Melasniemi, 1988). Molecular sieve chromatography on Sephadex G-100 (Takasaki, 1976b; McCleary and Anderson, 1980; Plant *et al.*, 1986) and Sephadex G-200 (Nakamura *et al.*, 1975; Saha *et al.*, 1979; Ohba and Ueda, 1973; Kuriki *et al.*, 1988a) has been employed for the separation of the respective pullulan-hydrolysing enzymes. Preparative isoelectric focusing has been reported only for the purification of glucoamylase I from *A. oryzae* (Saha *et al.*, 1979).

Affinity chromatography using α-cyclodextrin (schardinger dextrin) was reported for the selective isolation of pullulanase from *K. pneumoniae* by Enevoldsen *et al.* (1977), as well as for the neopullulanase from *B. stearothermophilus* TRS 40 (Kuriki *et al.*, 1988). The amylopullulanase from *Thermus* sp. AMD 33 was isolated by employing the same affinity matrix for the purification (Nakamura *et al.*, 1989). Saha *et al.* (1988) reported that pullulan covalently coupled to Sepharose CL-6B could separate the amylopullulanase of

Clostridium thermohydrosulfuricum strain Z 21-109. Keeping abreast with the recent trends in enzyme purification, fast protein liquid chromatographic separation (Pharmacia Superose-6 and/or Superose-12 columns) was reported for the purification of amylopullulanases from *C. thermohydrosulfuricum* E 101-69 (Melasniemi, 1988) and *C. thermosulfurogenes* EM1 (Spreinat and Antranikian, 1990).

1.3.3.1 Molecular weight of pullulan-hydrolysing enzymes

The molecular weights of various enzymes capable of pullulan hydrolysis have been summarized in Table 1.2. Most of the pullulan-hydrolysing enzymes, with the exception of amylopullulanases showed molecular weights in the range 30,000 to 100,000 daltons. A considerable variation was observed in case of amylopullulanases which showed molecular weights in the range of 100,000 - 500,000 daltons.

The molecular weights of pullulanases from different *K. pneumoniae* isolates showed a lot of variation, which could possibly be either due to the difference in the source or the multiplicity of the pullulanases. In general, the intracellular enzyme from *K. pneumoniae* appeared to be of a higher molecular weight than the extracellular enzyme, which indicated the presence of a signal peptidase processing step in the secretion of pullulanase from *K. pneumoniae* (Katsuragi *et al.*, 1987; Pugsley *et al.*, 1986). None of the pullulanases reported so far appear to have a sub-unit structure.

Table 1.2 Molecular weight of pullulan-hydrolysing enzymes

Organism	Molecular Weight	Reference
I. Pullulanase (Pullulan 6-glucanohydrolase, EC 3.2.1.41)		
1. *Aerobacter aerogenes* No. 105		
Extracellular	58,000	Ueda and Ohba, 1972
Intracellular	90,000	Ohba and Ueda, 1973
2. *A. aerogenes* P1		
Intracellular	56,000	Mercier *et al.*, 1972
Intracellular	more than 500,000	- „ -
3. *Klebsiella pneumoniae*		
Extracellular	126,000	Brandt *et al.*, 1976
Protease-released	141,000	- „ -
4. *Bacillus acidopullulyticus*		
F1	115,000	Kusano *et al.*, 1988
F2	116,000	- „ -
5. *Thermoactinomyces thalpophilus* No. 15	79,000	Odibo and Obi, 1988
6. *Bacillus cereus* var. *mycoides*	110,000	Takasaki, 1976b
7. *Bacillus* sp. No. 202-1	92,000	Nakamura *et al.* 1975
8. *Bacillus stearothermophilus* TRS 128	83,000	Kuriki *et al.*, 1988b

Table 1.2 contd .....

Organism	Molecular Weight	Reference
II Isopullulanase (Pullulan 4-glucanohydrolase, EC 3.2.1.57)		
1. *Aspergillus niger*	74,000	Sakano *et al.*, 1972
III Neopullulanase		
1. *B. stearothermophilus* TRS 40	62,000	Kuriki *et al.*, 1988a
IV Glucoamylase (1,4- α -glucan glucohydrolase, EC 3.2.1.3)		
1. *Aspergillus oryzae*	87,000	Saha *et al.*, 1979
2. *Hormoconis resinae* ATCC 20495		
Glucoamylase P	71,000	Fagerström *et al.*, 1990
Glucoamylase S	78,000	
3. *Candida antarctica* CBS 6678	48,000	DeMot and Verachtert, 1987
4. *Saccharomyces cerevisiae* NCYC 625	250,000	Kleinman *et al.*, 1988
5. *Saccharomyces diastaticus* 5206-1B	300,000	Modena *et al.*, 1986
6. *Aspergillus niger* IMDCC No. 1203	63,000	Fogarty and Benson, 1983

Table 1.2 contd.....

Organism	Molecular Weight	Reference
V Amylopullulanase		
1. *Thermoanaerobium* Tok6-B1	120,000	Plant *et al.*, 1987a
2. *Clostridium thermosulfurogenes* EM1	102,000	Spreinat & Antranikian, 1990
3. *Bacillus subtilis* TU	450,000	Takasaki, 1987
4. *Bacillus circulans* F-2	220,000	Sata *et al.*, 1989
5. *Thermoanaerobacter* strain B6A	450,000	Saha *et al.*, 1990
6. *Thermus aquaticus* YT-1	80,000	Plant *et al.*, 1986
7. *Bacillus* sp. 3183	190,000	Saha *et al.*, 1990
8. *Clostridium thermohydrosulfuricum* mutant Z 21-109	136,500	Saha *et al.*, 1988
9. *C. thermohydrosulfuricum* strain E 101-69		
Form I	370,000	Melasniemi, 1988
Form II	330,000	- „ -
10. *Thermus* sp. AMD 33	135,000	Nakamura *et al.*, 1989

Note : Unless otherwise mentioned, the enzymes are extracellular

1.3.3.2 Isoelectric pH (pI) of pullulan-hydrolysing enzymes

The isoelectric points of a majority of pullulan-hydrolysing enzymes have been reported in the literature. Ohba and Ueda (1975) reported three forms each of extracellular (pI's 3.72, 4.35, 7.7) and intracellular (pI's 3.88, 4.46, 7.6) pullulanases from *K. pneumoniae* using preparative scale column isoelectric focusing. In case of *Bacillus* sp. no. 202-1, an extremely acidic pI of less than 2.5 was reported by Nakamura *et al.*, (1975). Recently, Kusano *et al.* (1988) isolated two closely related forms of pullulanases from the commercial pullulanase preparation (Promozym 200L, Novo Industri A/S, Denmark) obtained from *B. acidopullulyticus*. The pI's of these two enzyme forms were found to be very similar (F1 = 5.0; F2 = 5.2).

A pI of 5.20 ± 0.15 was noted for the neopullulanase from *Thermoactinomyces vulgaris* (Shimizu *et al.*, 1982).

With the notable exception of the glucoamylase from *Candida antartica* CBS 6678 (pI = 10.1) (DeMot and Verachtert, 1987), acidic isoelectric points are reported for the glucoamylases (range of 3.5 - 4.5) capable of pullulan hydrolysis. These are *H. resinae* (pI = 3.8) (Fagerström *et al.*, 1990); *Saccharomyces cerevisiae* (pI = 4.62) (Kleinman *et al.*, 1988) and *A. niger* (pI = 4.2) (Fogarty and Benson, 1983).

Data on the isoelectric points of amylopullulanases is not available in all the reports. A pI of 4.13 has been reported for the amylopullulanase from *B. circulans* F-2 (Sata *et al.*, 1989). Similarly isoelectric points were reported for the enzymes from *Thermoanaerobacter* strain B6A (pI = 4.5) (Saha *et al.*, 1990), *C.*

thermohydrosulfuricum E 101-59 (pI = 4.25) (Melasniemi, 1988) and *Thermus* sp. AMD 33 (pI = 4.2) (Nakamura *et al.*, 1989). Saha *et al.* (1988) have reported an isoelectric point of 5.9 for the amylopullulanase from *C. thermohydrosulfuricum* Z 21-109.

1.3.3.3 Optimum pH of pullulan-hydrolysing enzymes

Most of the pullulan-hydrolysing enzymes reported so far showed an optimum activity in the pH range of 4.5 - 6.5. Notable exceptions were the pullulanases from *K. pneumoniae* (Mercier *et al.*, 1973) and *Thermoactinomyces thalpophilus* (Odibo and Obi, 1988), which showed optimum activity at pH 7.0. Pullulanases (F1 and F2) isolated from *B. acidopullulyticus*, an organism selectively isolated for the production of an acid stable pullulanase, showed optimum activity at pH 5.0 (Kusano *et al.*, 1988). An alkaline pullulanase isolated from *Bacillus* sp. 202-1, on the other hand, showed optimum activity between pH 8.5 - 9.0 (Nakamura *et al.*, 1975).

The isopullulanase from *A. niger* was active at an acidic pH of 3.0 - 3.5 (Sakano *et al.*, 1972), whereas the enzyme from *A. globiformis* T6 was optimally active at pH 4.8 (Okada *et al.*, 1988).

Neopullulanase from *B. stearothermophilus* TRS 40 showed an optimum activity at pH 6.0 (Kuriki *et al.*, 1988a). In *B. stearothermophilus* KP 1064 a pH optimum of 5.8 was observed by Suzuki and Imai (1985).

Glucoamylases were reported to hydrolyse pullulan optimally in the acidic range. The optimum pH for the hydrolysis of pullulan was reported to be

between 5.0 - 6.0 in case of *A. oryzae* (Saha *et al.*, 1979), between 4.0 - 6.0 for *C. antartica* CBS 6678 (DeMot and Verachtert, 1987), and around pH 3.5 - 4.0 for the glucoamylase P from *C. resinae* (McCleary and Anderson, 1980).

Most of the amylopullulanases were, in general, active in an acidic pH range (5.0 - 6.5) for the hydrolysis of starch as well as pullulan (Antranikian, 1990).

1.3.3.4 Optimum temperature of pullulan-hydrolysing enzymes

Most of the pullulanases from the mesophilic bacteria have a temperature optima between 50 - 60°C (Nakamura *et al.*, 1975; Takasaki, 1976b; Kusano *et al.*, 1988). The pullulanase from *T. thalpophilus* has a high temperature optimum of 70°C (Odibo and Obi, 1988).

K. pneumoniae pullulanase (Mercier *et al.*, 1973) and the isopullulanase from *A. niger* (Sakano *et al.*, 1972) were found to be optimally active at a comparatively low temperature (30°C).

The neopullulanase of *B. stearothermophilus* TRS 40, a thermophile, was optimally active at a temperature between 60 - 65°C (Kuriki *et al.*, 1988a). Similarly, the neopullulanase of *T. vulgaris* showed optimum pullulan hydrolysis at 70°C (Shimizu *et al.*, 1982).

Most of the glucoamylases active on pullulan had temperature optima of around 50°C, though they hydrolysed starch optimally at 60 - 65°C (McCleary and Anderson, 1980; DeMot and Verachtert, 1987). This difference in the temperature optima of hydrolysis was attributed by McCleary and Anderson (1980) to the presence of more reducing end groups in starch, which probably stabilize

the enzyme against inactivation.

In most of the cases, the amylopullulanases had high temperature optima in the range of 60 - 90°C (Antranikian, 1990).

1.3.3.5 pH and temperature stability of pullulan-hydrolysing enzymes

Stability of the pullulan-hydrolysing enzymes towards pH and temperature has received a lot of attention because of their commercial potential (Kennedy *et al.*, 1988; Saha and Zeikus, 1989). Most of the pullulanases (EC 3.2.1.41) are stable only upto 50 - 55°C. Extracellular pullulanase from *K. pneumoniae* was stable up to 40°C for 30 min over a wide range of pH (5.0 - 11.5) (Ohba and Ueda, 1972). The pullulanases reported from *Bacillus* sp. were stable up to 50°C (Takasaki, 1976b; Nakamura *et al.*, 1975). Enzymes from *B. stearothermophilus* TRS 128 (stable at 65°C for 60 min at pH 6.5) (Kuriki *et al.*, 1988b) and *B. acidopullulyticus* (stable up to 55°C at pH 5.0) (Kusano *et al.*, 1988) were relatively thermostable. Similar stability profiles were observed for the neopullulanase from *B. stearothermophilus* TRS 40 also (Kuriki *et al.*, 1988a). Pullulanases described so far were observed to be inactivated rapidly above 70°C.

Most of the glucoamylases which hydrolyse pullulan were stable up to 50°C (McCleary and Anderson, 1980; Saha *et al.*, 1979; Fogarty and Benson, 1983). The amylopullulanases from *Thermoanaerobium* Tok6-B1 (Plant *et al.*, 1987) and *T. aquaticus* (Plant *et al.*, 1986) were extremely thermostable (70°C for several hours between a pH range of 4.5 - 5.5). Enzymes from *Thermoanaerobacter* strain B6A (Saha *et al.*, 1990) and *Bacillus* sp. 3183 (Saha *et al.*, 1989) were found to be stable

up to 70°C. Notable exceptions to this class of enzymes were from *B. subtilis* TU (Takasaki, 1987) and *B. circulans* (Sata *et al.*, 1989) which were found to be stable only up to 50°C.

1.3.3.6 Effect of metal ions on pullulan hydrolysing enzymes

Silver, copper, mercury and lead are the principal metal ions which have been shown to be inhibitory for the activity of these enzymes even at concentrations of the order of 10^{-3} - 10^{-4} M. Ohba and Ueda (1975) have studied the effect of various metal ions on pullulanase from *K. pneumoniae*. Metal ions such as Co^{++}, Ni^{++}, Cd^{++}, Zn^{++} (each at 10^{-3} M) and Ca^{++} (at 10^{-2} M) inhibited the enzyme activity to a varying degree. However, Ca^{++} at 10^{-3} M concentration stimulated the activity of the enzyme. Calcium and barium ions were also found to stimulate the pullulanase from *B. stearothermophilus* TRS 128 (Kuriki *et al.*, 1988b). Pullulanase from *B. cereus* var. *mycoides* was found to be inhibited (40-85% inhibition) by Hg^{++}, Ag^{++}, Fe^{++} at a concentration of 10^{-3} M (Takasaki, 1976b). Similar results were observed with pullulanase from *Bacillus* sp. 202-1 by Nakamura *et al.* (1975).

Isopullulanase from *Arthrobacter globiformis* T6 was found to be inhibited in the presence of Hg^{++} and Fe^{+++} at 5 X 10^{-3} M concentration (Okada *et al.*, 1988).

Pullulan hydrolase (an enzyme that resembles neopullulanase) from *B. stearothermophilus* KP 1064 showed complete inhibition of the activity by Hg^{++} and Cd^{++} (2 X 10^{-3} M) (Suzuki and Imai, 1985).

In case of amylopullulanase from *Thermoanaerobium* Tok6-B1, Ca^{++} (10^{-3} M) was apparently required for the activity of the enzyme (Plant *et al.*, 1987). Requirement of calcium ions for the stabilization was also noted both for *Bacillus* sp. 3183 (Saha *et al.*, 1989) and *C. thermohydrosulfuricum* E 101-69 (Melasniemi, 1988). With the exception of some of these amylopullulanases, none of the other pullulan-hydrolysing enzymes reported to have a requirement for metal ions.

1.3.3.7 Nature of pullulan-hydrolysing enzymes

There are very few reports regarding the nature of modification (e.g. glycosylation, liposylation etc.) in case of pullulan-hydrolysing enzymes. The pullulanase from *K. pneumoniae* was shown to be a lipoprotein by Pugsley *et al.* (1986). The enzyme was shown to carry fatty acyl modified cysteine residues at its amino terminal end.

The glucoamylases from *Trichosporon pullulans* (DeMot and Verachtert, 1986) and the amylopullulanase from *C. thermohydrosulfuricum* Z 21-109 (Saha *et al.*, 1988) were shown to have glycosyl side chains associated with the enzyme proteins. Chemical characterization of these side chains has not been reported so far.

1.3.3.8 Inhibition of pullulan-hydrolysing enzymes

Marshall (1973) first identified schardinger dextrins (cyclomaltooligosaccharides) as potent inhibitors of pullulanase from *K. pneumoniae*. It was reported that cyclohexaamylose (α-cyclodextrin) and cycloheptaamylose (β-cyclodextrin) inhibited the pullulanase from *K.*

pneumoniae. At the same time it was demonstrated that the corresponding linear oligosaccharides had no effect. It was shown in the same report that the inhibition with schardinger dextrins appeared to be specific for pullulanase, with other starch-hydrolysing enzymes being hardly affected at all (Marshall, 1973). Apart from the report of schardinger dextrins, there are no reports of any other compounds which selectively inhibit pullulanases.

The specificity of schardinger dextrins towards pullulanases has been successfully used for the affinity purification of pullulanase from *K. pneumoniae* (Enevoldsen *et al.*, 1977), as well as, for the neopullulanase from *B. stearothermophilus* TRS 40 (Kuriki *et al.*, 1988a). Recently they have also been used for the amylopullulanase from *Thermus* sp. AMD 33 (Nakamura *et al.*, 1989).

Acarbose is a specific inhibitor of α-glucosidases produced by a *Actinoplanes* sp. (Schmidt *et al.*, 1977). It has been shown that acarbose acts as a strong competitive inhibitor of the glucoamylases and was succseefully used to construct an affinity matrix for the selective isolation of glucomaylases from *A. niger* (Ono and Smith, 1986) and *Candida antartica* CBS 6678 (DeMot and Verachtert, 1987). The inhibitor constant for acarbose was reported to be of the order of 1×10^{-7} M for the glucoamylase from *C. antartica* CBS 6678 (DeMot and Verachtert, 1987).

Para-chloromercury benzoic acid (p-CMB) (2×10^{-3} M) was shown to inhibit the pullulanase from *B. cereus* var. *mycoides* to an extent of 50% (Takasaki, 1976b). A similar observation was also made for the neopullulanase from *T. vulgaris* by Shimizu *et al.* (1982). Suzuki and Imai (1985) found that the neopullullanase

from *B. stearothermophilus* KP 1064 was almost completely inhibited by the action of p-CMB (4.5 X 10^{-7} M). Mono-iodo acetic acid, N-bromosuccinimide (NBS) and phenyl methane sulfonyl fluoride (PMSF) were shown to have no effect on the activity of *T. vulgaris* enzyme at a concentration of 10^{-3} M (Shimizu *et al.*, 1982).

In case of the amylopullulanase from *Thermoanaerobacter* strain B6A, NBS (10^{-4} M) completely inactivated the enzyme. β-Cyclodextrin (32% inhibition) and γ-cyclodextrin (48% inhibition) were significant inhibitors (10^{-2} M) of pullulan hydrolysis in case of amylopullulanase from *Thermoanaerobacter* strain B6A. However, at the same concentration α-Cyclodextrin had no inhibitory effect on the enzyme activity (Saha *et al.*, 1990).

1.3.3.9 Transglycosylation activity of pullulan-hydrolysing enzymes

Many of the carbohydrate hydrolysing enzymes show a reversal of their normal catalytic action (transglycosylation) under a high concentration of their end-products. This results in the production of higher oligosaccharides from their normal end-products because of the transglycosylation reactions (Reese *et al.*, 1968; Pazur and Okada, 1967). A similar capability of pullulanase to catalyze transglycosylation was first demonstrated by Abdullah and French (1966). They observed that from a solution of maltotriose, pullulanase synthesized oligosaccharides having R_f corresponding to compounds containing one or two α-(1->6)-glucosidic linkages on paper chromatography. French and Abdullah (1966) reported that with maltose as a substrate, two tetrasaccharides could be detected in the reaction mixture. These were 6^2-α-maltosylmaltose and

6^1-α-maltosylmaltose. Similarly, with maltotriose as a substrate, pullulanase synthesized three hexasaccharides characterized as (6^1-,6^2-,6^3-maltotriosylmaltotriose).

1.3.3.10 Substrate specificity of pullulan-hydrolysing enzymes

To understand the mode of action of various pullulan-hydrolysing enzymes, the substrate specificity of these enzymes was investigated. The specificity of the enzyme catalysis is observed either to be due to the linkage specificity, the glycosyl group requirements or the chain length of the substrate.

a. *Pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41)*

Wallenfels *et al.* (1965) initially demonstrated that with pullulan as a substrate, pullulanase quantitatively hydrolysed it to maltotriose. The substrate specificity of the pullulanase from *K. pneumoniae* was extensively investigated by Abdullah and French (1970) using a number of starch oligosaccharides. Their studies confirmed the linkage specificity of pullulanase and found that it has been restricted to α-(1->6)-linkages only. The α-(1->4)-linkages in all the oligosaccharides tested were not attacked by the enzyme. The minimum chain-length of the substrate amenable to the hydrolysis by pullulanase was determined by Abdullah and French (1970) to be a tetrasaccharide made up of two maltose units linked through an α-(1->6)-linkage on the terminal glucose residues. Two forms, 6^2-maltosylmaltose or 6^1-maltosylmaltose could be hydrolysed by the enzyme. Starch oligosaccharides and different dextrins containing two or more

α-(1->6)-linkages could also be cleaved by pullulanase. Similar results were subsequently reported for a number of pullulanases (Walker, 1968; Nakamura *et al.*, 1975; Takasaki, 1976b; Kuriki *et al.*, 1988b; Kusano *et al.*, 1988).

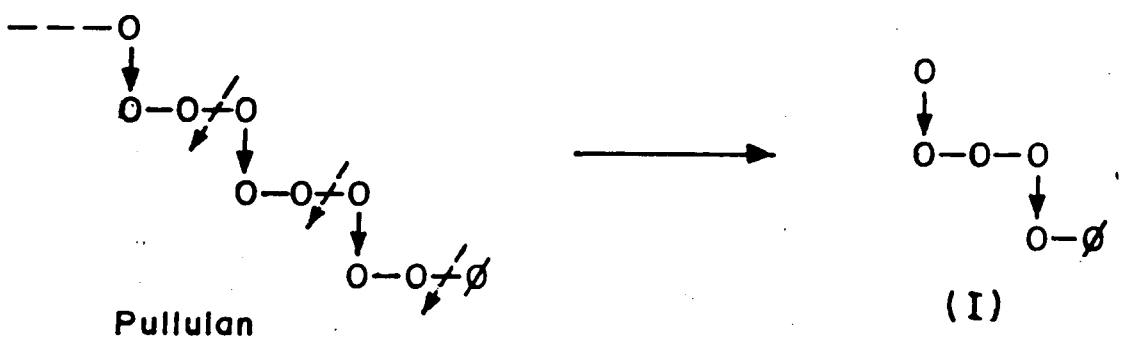
b. *Isopullulanase (pullulan 4-glucanohydrolase, EC 3.2.1.57)*

Investigating the substrate specificity of isopullulanase, Sakano *et al.* (1971,1972) reported the production of isopanose as the sole product of pullulan hydrolysis. From pullulan, the enzyme produced a mixture of isopanose, isomaltose, maltose and glucose as the end products. The enzyme was also reported to act on glycogen β-limit dextrins and branched dextrins producing glucose (Sakano *et al.*, 1972). Isopullulanase from *A. globiformis* T6 also formed isopanose as the sole product of hydrolysis of pullulan (Okada *et al.*, 1988).

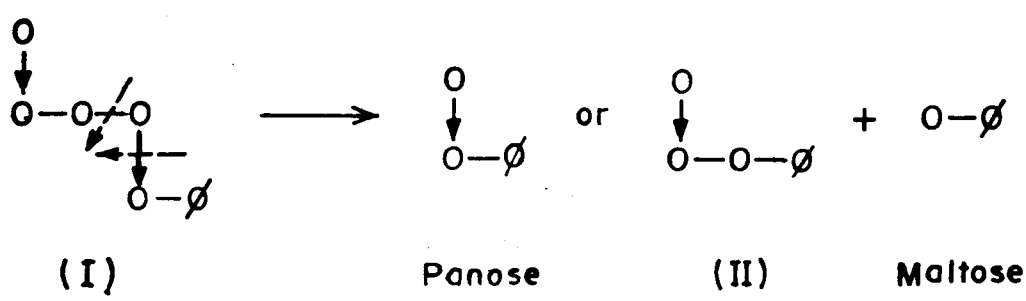
c. *Neopullulanase*

Neopullulanase was found to hydrolyse primarily pullulan. Little hydrolysis of starch was detected after prolonged incubation (24 h) with the formation of glucose and maltose (Kuriki *et al.*, 1988a). Imanaka and Kuriki (1989) have investigated the mechanism of hydrolysis of pullulan by this enzyme using a range of oligosaccharides. Based on these results, a three step reaction sequence was proposed for the action of neopullulanase on pullulan (Imanaka and Kuriki, 1989).

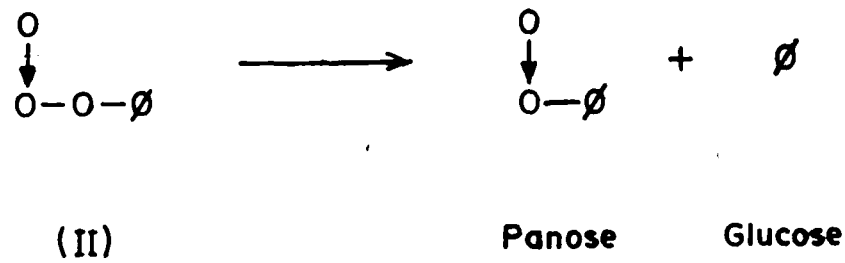
Step I: Neopullulanase attacks only the α-(1->4)-glucosidic linkages on the non-reducing end of the α-(1->6)-linkages in pullulan at random and produces panose and several other intermediate products like, 6^2-o- α-(6^3-o- α-glucosyl-maltotriosyl)-maltose [I], which is attacked in the step II of the reaction sequence.



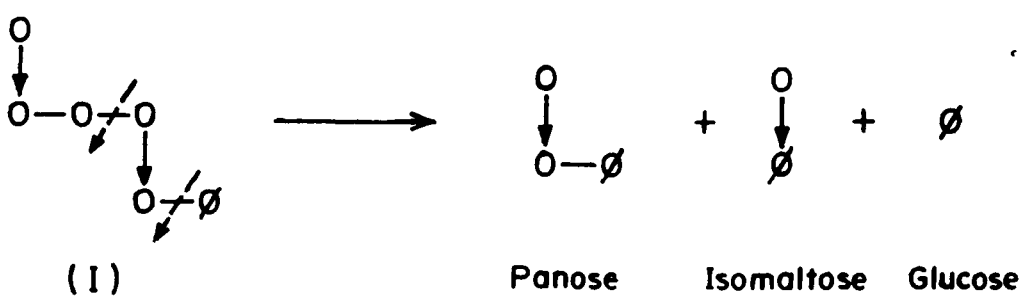
Step II: the enzyme attacks either the α-(1->4)- or α-(1->6)-glucosidic linkages in [I] and produces either panose, 6^3- α-glucosyl-maltotriose and maltose



Step III: 6^3-o- α -glucosyl maltotriose [II] is hydrolyzed to produce panose and glucose



The product [I] from step I, may be hydrolysed to produce a little amount of isomaltose and glucose along with panose.



d. *Glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3)*

Glucoamylases hydrolysed pullulan molecule from the non-reducing end producing glucose as the sole product of hydrolysis (Saha *et al.*, 1979; McCleary and Anderson, 1980; DeMot and Verachtert, 1987).

e. *Amylopullulanases*

Amylopullulanases are reported to have a dual substrate specificity (Saha and Zeikus, 1989; Antranikian, 1990). The product of hydrolysis of pullulan with these enzymes exclusively appeared to be maltotriose (Plant *et al.*, 1986; Plant *et al.*, 1987; Melasniemi, 1988; Saha *et al.*, 1988; Spreinat and Antranikian, 1990).

In case of hydrolysis of starch, these enzymes exhibit a mechanism of reaction analogous to α -amylase (Saha and Zeikus, 1989). Starch is converted to DP2 - DP5 oligosaccharides by most of the enzymes reported (Plant *et al.*, 1986; Plant *et al.*, 1987; Melasniemi, 1988; Saha *et al.*, 1989; Saha *et al.*, 1990). The enzyme from *B. subtilis* TU appeared to be an exception to this observation, wherein maltose (DP2) appeared as the major product of hydrolysis of amylose and soluble starch (Takasaki, 1987).

1.3.4 Applications of pullulan-hydrolysing enzymes

1.3.4.1 Starch hydrolysis

Starch is the principal reserve polysaccharide of the plant kingdom and is mostly found in the seeds of maize, wheat, barley, oat, rice, sorghum and corn. It is a mixture of linear (amylose) and branched (amylopectin) homopolymers of D-glucose. Amylose is made up of linear chains of α -D-glucopyranose units linked by α -(1->4)-linkages with a degree of polymerization of 1×10^2 - 4×10^5. Amylopectin, contains short linear chains (DP 17 - 23) of α -(1->4)-linked, α -D-glucopyranose residues joined by α -(1->6)-linkages to form a highly branched structure (DP 1×10^4 - 4×10^7). The degree of branching and the ratio

of amylose to amylopectin is found to vary with the source (Kennedy *et al.*, 1988).

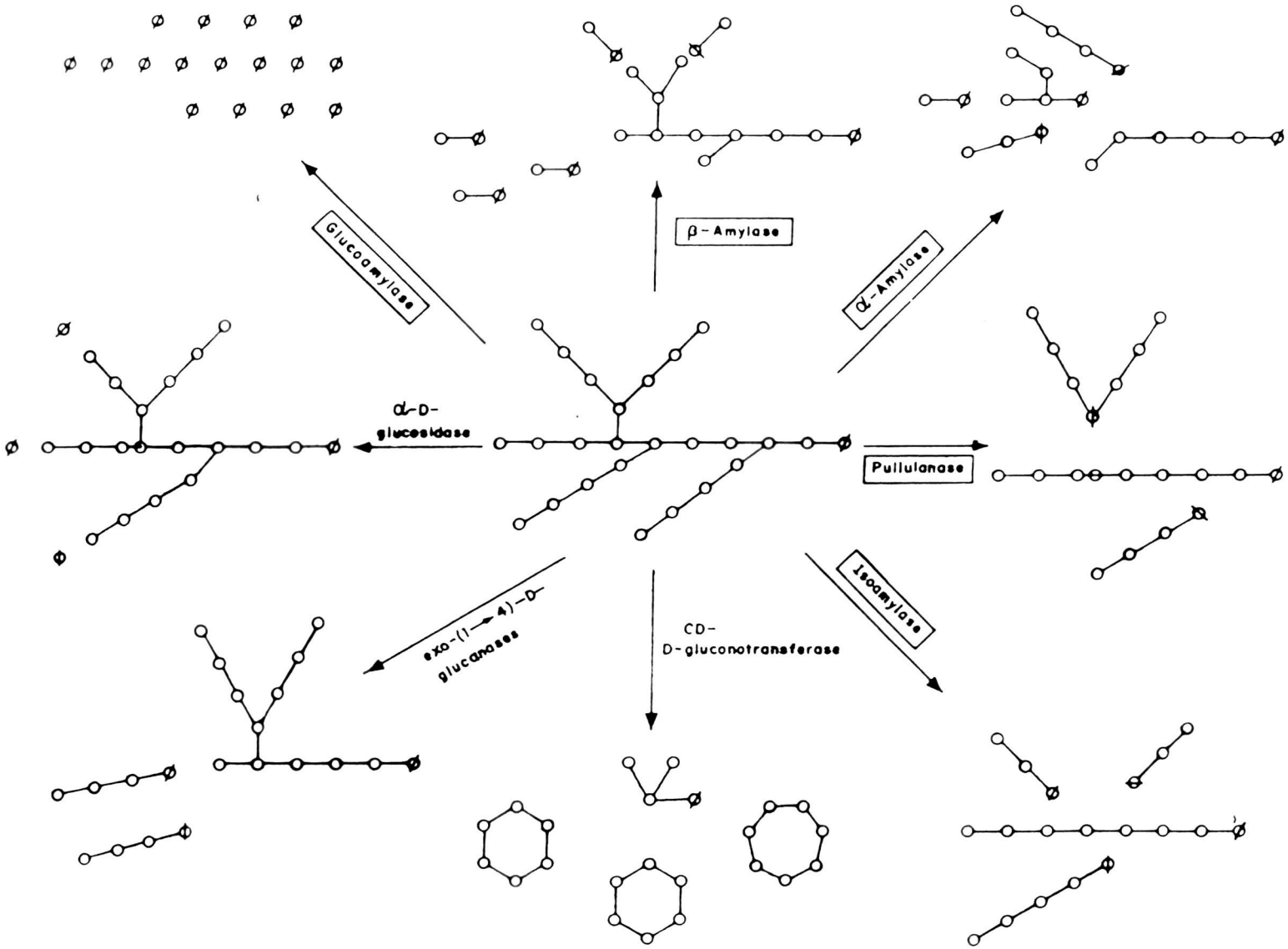
The conventional method of acid-hydrolysis of starch to produce glucose syrups for the various food industries has now largely been superceded by the use of starch-hydrolysing enzymes. Enzymatic hydrolysis of starch has been found to give higher yields of more specific hydrolysates of starch as compared to the conventional acid hydrolysis. This was also found to result in less number of by-products (Norman, 1981). A number of starch hydrolysing enzymes are presently known, but only a few have commercial significance. Schematically the action of these enzymes are presented in Fig. 1.1.

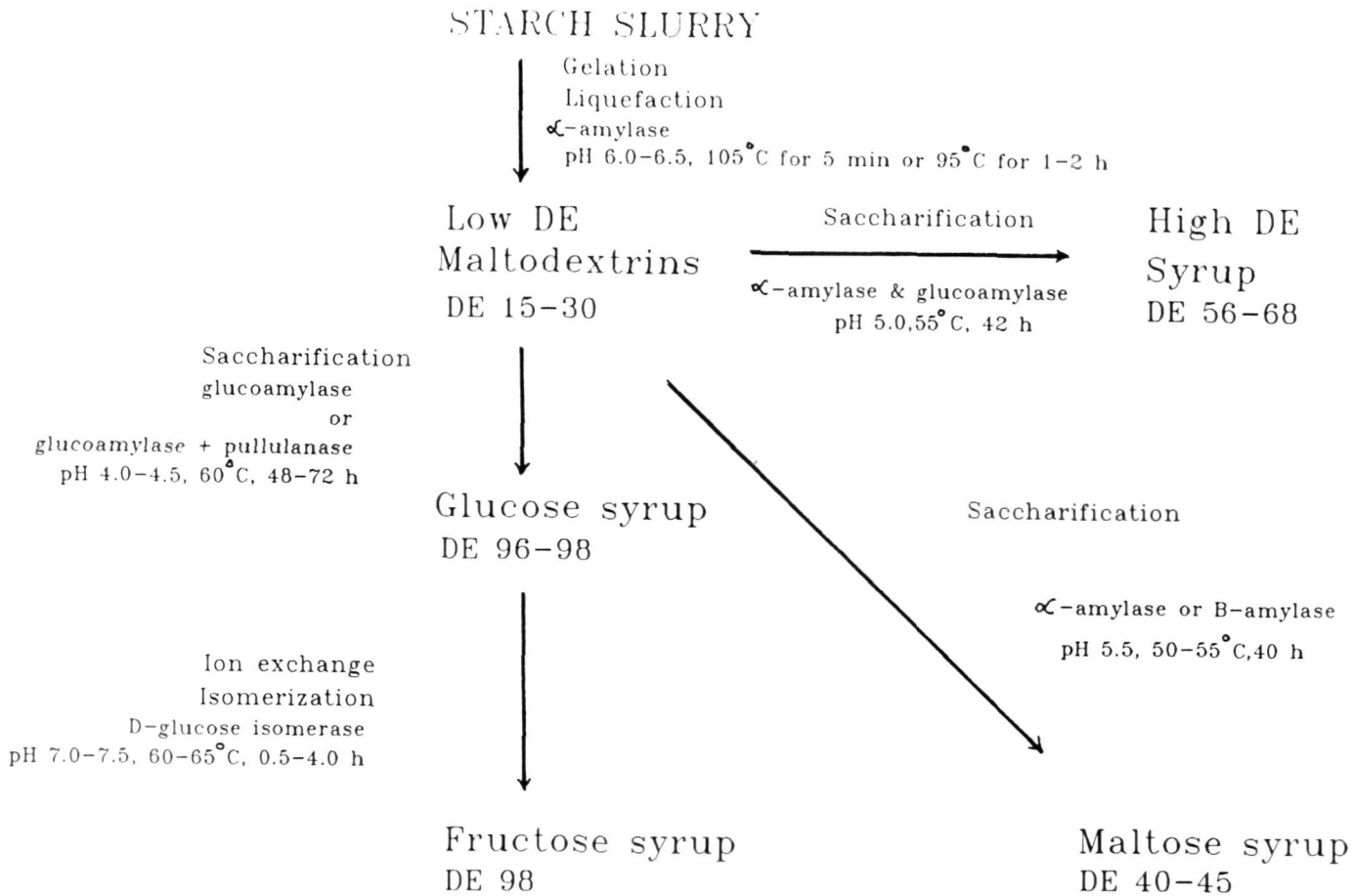
Various strategies of enzymatic hydrolysis of starch have been presented in Scheme 1.

a. *Glucose syrups*

If a debranching enzyme and glucoamylase are used simultaneously to saccharify starch, increased glucose yields can be achieved. In the absence of a suitable debranching enzyme, the α-limit dextrins formed by the action of α-amylase during the liquefaction of starch hamper glucoamylase action in the subsequent step of saccharification. Therefore, for the complete conversion of starch to glucose more time is required. If a suitable debranching enzyme like pullulanase or isoamylase is present during the saccharification, hydrolysis of the limit-dextrins reduces the time period of the whole process. This not only saves the amount of glucoamylase needed per gram of starch hydrolyzed but also reduces the time required for the complete hydrolysis. Norman (1981) reported that less

Fig. 1.1: Enzymes capable of hydrolysis of starch.


Glucoamylase
β-Amylase
α-Amylase
α-D-
glucosidase
Pullulanase
exo-(1→4)-D-
glucanases
CD-
D-gluconotransferase
Isoamylase



Scheme 1

Industrial enzymatic processing of starch
(Modified from Kennedy et al. ,1988)

amount of glucoamylase used resulted in lesser amount of reversal product formation (e.g. isomaltose) and, inturn, the effective dextrose levels achieved were higher.

Saha and Zeikus (1989) reported that in the saccharification of starch, pullulanase increased the concentration of glucose (by about 2%, in combination with glucoamylase) or maltose (by about 20-25 %, in combination with β-amylase). It also reduced the reaction time required for the complete hydrolysis of starch from 72 to 48 h. This has, inturn, allowed an increase in the substrate concentration used (to 40% DS). At the same time the amount of glucoamylase required in the reaction could be reduced by up to 50% to achieve the same degree of hydrolysis.

Isoamylases are better debranching enzymes than pullulanases because they have a much higher activity towards amylopectin (Norman, 1981). In addition, their pH optima are between 4.0 - 4.5, which makes them compatible with glucoamylases for the saccharification process. However, most of the isoamylases tested so far suffered a serious drawback from the process point of view. They were observed to be thermolabile and therefore one had to carry out the saccharification at a lower temperature (less than 50°C), which was not commercially feasible (Norman, 1981).

Novo Nordisk BioLabs (Denmark) commercially produces a pullulanase preparation (Promozym 200L), derived from *Bacillus acidopullulyticus* which is optimally active around a pH of 5.0 and 60°C, making it compatible with the present starch hydrolytic processes.

b. *Maltose syrups*

Maltose syrups are important because of their specific characteristics such as low sweetness (compared to the glucose syrups), low viscosity and hygroscopicity in solution and thermostability.

Treatment of starch with maltogenic exo-amylases such as barley β-amylase alone, resulted in the formation of maltose syrups containing 60% maltose in 48 h. However, in the presence of a debranching enzyme, like *K. pneumoniae* pullulanase or the isoamylase from *Pseudomonas amylodermosa*, the maltose levels achieved in the syrups could reach 80% in the same time period (Norman, 1981).

A continuous maltose producing system using porous chitosan beads for immobilization of β-amylase and pullulanase has been recently described by Yoshida *et al.* (1989). Maltose levels of the order of 70 - 80% maltose were achieved by keeping the concentration of the starch feed below 25% (w/w). A maltose yield of more than 65% could also be maintained for over 500 h (Yoshida *et al.*, 1989).

1.3.4.2 Production of branched cyclic oligosaccharides

While investigating the substrate specificity of pullulanase from *K. pneumoniae*, Abdullah and French (1970) reported the presence of transglycosylation activity of pullulanase. Interestingly, this transglycosylation activity has been used to produce a variety of specific oligosaccharides (Kitahata *et al.*, 1987; Shiraishi *et al.*, 1989; Hizukuri *et al.*, 1989; Hisamatsu and Yamada, 1989). Using cyclodextrins and mono- and di-saccharide sugars as substrates,

corresponding branched cyclodextrins were principally obtained in these reactions.

Kitahata *et al.* (1987) reported successful synthesis of 6-o-α-maltosylcyclomaltohexaose (G_2-cG_6), 6-6'-di-o- α- maltosylcyclomaltohexaose [(G_2)$_2$-cG_6] etc. using the pullulanases from *K. pneumoniae* and *B. acidopullulyticus*. It was found that the pullulanase from *B. acidopullulyticus* was more efficient in carrying the above mentioned reactions as compared to the pullulanase from *K. pneumoniae* and the isoamylase from *P. amylodermosa* (Kitahata *et al.*, 1987). Similar reactions were also reported by Shiraishi *et al.* (1989) and Hizukuri *et al.* (1989) for the synthesis of modified cyclodextrins using pullulanase.

Hisamatsu and Yamada (1989) have achieved a continuous production of maltosyl-cyclomaltoheptaose (G_2-G_7) using pullulanase from *B. acidopullulyticus* immobilized on partially deacylated chitin (PDAC-35) in a bioreactor. Similar observations have also been made by Kusano *et al.* (1989) by immobilizing pullulanase on porous glass, glutaraldehyde-treated chitosan beads and Amberlite IRC-50.

The amylopullulanases are being projected as novel enzymes which can generate entirely new processes for the starch industry. These enzymes can be used to hydrolyse starch in a single step process to syrups of maltoologosaccharides with DP between 2 and 5, since the liquefaction as well as the saccharification processes can potentially be carried out by the same enzyme. The optimum pH (~ 5 - 5.5)

of these enzymes is compatible with most of the present day starch-hydrolysis processes. Therefore, these enzymes can also be used as useful supplements for the exsisting starch hydrolysis processes (Saha and Zeikus, 1989).

1.4 PRESENT INVESTIGATION

The enzymatic saccharification of starch to glucose is one of the most important commercial applications of microbial enzymes in use today (Fogarty, 1983; Kennedy *et al.*, 1988). In order to accelerate the enzymatic hydrolysis of starch by amylases, the supplementation of pullulanase has been found to be very useful (Kennedy *et al.*, 1988; Saha and Zeikus, 1989). Pullulan acts as a model polysaccharide mimicking the brach-points present in amylopectin, a constituent polymer of starch. Hence, the identification of the presence of pullulan-hydrolysing activity in microorganisms makes them a potential candidate for the production of starch debranching enzymes. There are very few reports of fungal cultures producing pullulan-hydrolysing enzymes (Saha *et al.*, 1979; McCleary and Anderson, 1980; DeMot and Verachtert, 1987). Therefore, it was of interest to isolate and characterize the pullulan-hydrolysing activity produced by *S. rolfsii*, a basidiomycetous fungus already well characterized for its cellulolytic enzymes in this laboratory (Lachke and Deshpande, 1988).

The work presented in this thesis includes the following investigations:

1. Optimization of the production of the pullulan-hydrolysing activity and some studies on the effect of additives on the thermostability of the enzyme (Chapter III).
2. Purification and the physico-chemical characterization of the enzyme activity (Chapter IV).
3. Rapid isolation and regeneration of *S. rolfsii* protoplasts and their potential application for starch hydrolysis (Chapter V).

CHAPTER II

MATERIALS AND METHODS

CHAPTER 2

2.1 MATERIALS

The materials and chemicals used in the present studies were purchased from the suppliers listed in Table 2.1.

2.2 METHODS

2.2.1 Organism:

Sclerotium rolfsii (N.C.I.M. 1084) (a sclerotial state of *Corticium rolfsii*) was used in the present work.

2.2.2 Cultivation and preparation of the culture filtrate of S. rolfsii

S. rolfsii (NCIM 1084) was grown on NM-2 medium developed by Sadana *et al.* (1979) replacing cellulose by soluble starch (1% w/v). The composition of the NM-2 medium is summarized in Table 2.2. The culture was incubated at 30°C for 10 days on a rotary shaker (200 rpm) in 500 ml Erlenmeyer flasks containing 100 ml of the growth medium. Prior to autoclaving at 121°C for 20 min, the pH of the medium was adjusted to 6.5 with ortho-phosphoric acid, unless otherwise mentioned. Stock cultures of the organism were maintained on potato-dextrose agar (PDA) slants and subcultured once every 4 weeks. The media were inoculated with mycelium directly from the 6 - 8 days old PDA slants. The culture was harvested on the 10th day by decantation of the supernatant and filtration through a pad of glass wool. The clear supernatant was used for the estimation of the enzyme activities after adjustment of

Table 2.1 : Chemicals and Materials used in the present study.

Chemicals / Materials	Suppliers
Pullulan PF-20	Hayashibara Biochemical Company, Japan
Pullulan, potato amylopectin, oyster glycogen, dextran, α -methyl-d-mannopyranoside, malto-oligosaccharides, tris buffer, β-alanine, maleic acid, fluorescein isothiocynate, wheat germ agglutinin, commassie brilliant blue G-250 and R-250, acrylamide, bis-acrylamide, bovine serum albumin, ovalbumin, lysozyme, soybean trypsin inhibitor, sodium dodecyl sulfate, SDS-prestained molecular weight marker kit	Sigma Chemical Company, St. Louis, U.S.A.
Bio-Gel P-150	Bio-Rad Laboratories, U.S.A.
DEAE-Cellulose DE-52	Whatman Biosystems Ltd., U.K.
Blue Dextran, Concanavalin A-Sepharose CL-4B, Ampholines (pH range 3.5 - 10.0, 2.5 - 4.0 and 4.0 - 6.0), Sephadex G-100	Pharmacia, Sweden
Glucose oxidase - peroxidase kits	
"GLOX"	Kabi Diagnostica, Sweden
"Glucoset"	Ranbaxy Chemical Company, India

Table 2.1 Contd .....

Chemicals / Materials	Suppliers
Malt extract, yeast extract, proteose peptone, bacto-agar	Difco Labotatories, U.S.A.
Potato starch, amylose	BDH Laboratories, U.K. and India
Dinitrosalicylic acid, 2-mercaptoethanol	Koch-Light Labotatories, Germany
Trichloroacetic acid, Folin-Ciaocalteau Reagent	SRL Laboratories Private Ltd., India
Tween-80 (Polyoxyethylene sorbitan monooleate)	SISCO Chemical Company, India
Soluble starch for growth medium	Connought Medical Laboratory, Toronto, Canada
Sodium alginate	Loba Chemical Company, India
N,N,N',N'-Tetramethyl methylene diamine	Eastman Kodak Chemical Company, U.S.A.
NovoZym 234	Novo Nordisk Biolabs, Denmark
Acarbose	A gift from Bayer AG, Germany
Rest of the chemicals	Analytical reagent grade

Table 2.2 : Composition of NM-2 medium for the cultivation of *S. rolfsii.*

Composition	$g.l^{-1}$
KH_2PO_4	2.00
$(NH_4)_2HPO_4$	7.00
Urea	0.30
$MgSO_4.7H_2O$	0.30
$CaCl_2.2H_2O$	0.30
Proteose peptone	0.25
Yeast extract	0.10
Tween-80	0.33 ml
Trace metal solution *	1.00 ml
Soluble starch	10.00
pH	6.5

* Trace metal solution contained ($mg.l^{-1}$) : $FeSO_4.7H_2O$, 5.0; $MnSO_4.7H_2O$, 1.56; $ZnSO_4.7H_2O$, 3.34; $CoCl_2$, 2.0.

the pH to 4.8 - 5.0 with tri-sodium citrate (1 M). The culture filtrate could be stored for long periods at 4°C or frozen in the presence of 0.01% sodium azide as a preservative, without any loss in enzyme activity.

2.2.3 Cultivation of S. rolfsii for the production of protoplasts

The liquid malt extract - yeast extract - glucose (MYG) medium was used for the preparation of mycelia for the isolation of protoplasts. The composition of the medium was as follows:

Malt extract	0.1 gm
Yeast extract	0.1 gm
Glucose	1.0 gm
Distilled water	100 ml
pH	**5.5**

The medium was directly inoculated with mycelium from PDA slants (5 - 6 days old). The flasks were then incubated on a rotary shaker (200 rpm) for 24 - 48 h at 30°C, unless otherwise mentioned.

2.2.4 Cultivation media for the regeneration of protoplasts

The medium used for the studies on regeneration of protoplasts was MYG medium supplemented with one of the following stabilizers: NaCl (0.7 M), KCl (0.6 M), $MgSO_4$ (0.6 M), $MgCl_2$ (0.7 M), NH_4Cl (0.4 M), sorbitol (0.4 M) and sucrose (0.6 M). Wherever required, these media were solidified using Difco Bacto-Agar (2% w/v, unless otherwise mentioned).

2.2.5 Induction medium for the studies on enzyme secretion by protoplasts

The induction medium used for the studies of enzyme secretion included 1% hydrolysed starch prepared in 0.05 M acetate buffer (pH 5.0), stabilized with 0.6 M KCl. The medium used for the studies of starch hydrolysis by protoplasts included 1% tapioca or potato starch instead of the hydrolysed starch.

All the media used in the present studies were sterilized by autoclaving at 121°C for 20 min. ,

2.2.6 Isolation of protoplasts

The mycelial growth (24 h) was harvested, washed twice with sterile distilled water and then once with 0.05 M maleic acid-NaOH buffer (pH 5.0) containing 0.6 M KCl (stabilized buffer). The mycelial clump was pressed between filter paper (Whatman No. 1) to remove excess water. A 5 $mg.ml^{-1}$ solution of NovoZym 234 (Novo Industri, A/S, Denmark) was prepared in the stabilized buffer and filter sterilized (0.45 μ filter, Millipore Interech Corporation Inc., U.S.A.). For the isolation of protoplasts, 300 mg of damp mycelium was incubated with 6 ml of NovoZym 234 in a 50 ml conical flask, on a reciprocating water bath shaker (30°C, 4 h, 75 $strokes.min^{-1}$) (AquaTherm, New Brunswick Scientific Company, U.S.A.). After completion of the enzymatic digestion, the suspension was filtered through a sterile sintered glass filter (Porosity G1, Jena, presaturated with stabilized buffer) to remove the mycelial debris. The protoplasts were collected by centrifugation (500 xg for 10 min), then gently resuspended and washed twice with the stabilized buffer. Integrity of the isolated protoplasts was checked by vital staining using eosin (0.1 % w/v, in stabilized buffer). Finally the protoplasts were counted on a haemocytometer.

2.2.7 Photomicrography

For the photomicrographic studies of protoplasts and their regeneration, the agar drop (AD) and the thin layer agar (TLA) methods described by Kobayashi *et al.* (1985) were followed. Fluorescence photomicrography was carried out on a Carl-Zeiss Fluoval microscope (filter conditions: excitation filter, KP 490; dichroic mirror, FT510 and barrier filter, G497). Fluorescein isothiocynate-Wheat Germ Agglutinin conjugate was prepared according to Marshall *et al.* (1958) for the specific detection of chitin in the cell wall of the regenerating protoplasts.

2.2.8 Immobilization of protoplasts

An aliquot of 9 ml of sodium alginate (2% w/v) containing 0.6 M KCl was thoroughly mixed with 1 ml of the protoplast suspension (1 - 2 X 10^7). The suspension was added drop-wise to a solution of $CaCl_2$ (2% w/v) containing 0.6 M KCl to form beads under mild agitation. The beads were left in $CaCl_2$ solution for 30 min and then washed thoroughly with stabilized buffer. The integrity of the free and the immobilized protoplasts was checked by using eosin (0.1 % w/v in stabilized buffer) staining.

2.2.9 Enzyme secretion pattern and the saccharification of starch by free and immobilized protoplasts

Immobilized protoplasts (6 - 7 g wet weight) were incubated with 10 ml of the appropriate starch suspension at 30°C on a reciprocating water bath shaker (75 strokes.min^{-1}). Samples (1 ml) were drawn at different time intervals for assays. An identical experiment was carried out with free protoplasts (1-2 X 10^7).

Operational stability was determined by the same procedure described above, with estimation of the glucose and the pullulan-hydrolysing activity after 48 h. After every cycle (48 h), the beads were washed with stabilized buffer and resuspended in fresh substrate.

2.2.10 Effect of various additives on the thermal stability of pullulan-hydrolysing activity from S. rolfsii

Pullulan-hydrolysing enzyme inactivation was measured by incubating the enzyme from culture filtrate that was lyophilized and then extensively dialysed against 0.05 M acetate buffer (pH 4.9) in presence of different additives at 60°C (400 μg protein.ml^{-1}, ~0.5 IU). The additives used included ethylene glycol, glycerol, xylitol, sorbitol (between 0 - 4 or 5 M final concentration) and pullulan (5 and 25 mg.ml^{-1}). At regular intervals, aliquots of the enzyme were removed from the test and immediately frozen. The remaining pullulan-hydrolysing activity was then estimated as described in section 2.3.6.1, but at 37°C instead of the usual 50°C. The initial enzyme activity (0 h) was considered as control (100%) and the residual activities in the samples expressed accordingly. Appropriate blank test, i.e. enzyme without any additive was processed in parallel in each experiment.

2.3 METHODS OF ANALYSIS

2.3.1 Determination of reducing sugars

Reducing sugars (RS) were measured in the aliquots of the reaction mixtures as glucose equivalents either by the dinitrosalicylic acid method (DNS) (Fischer and Stein, 1961) or by Somogyi's (1952) modification of the Nelson (1944) method (Somogyi-Nelson). Specific determination of glucose was carried out using

the glucose oxidase - peroxidase method (Bergmeyer *et al.*, 1983). On an equal weight basis the absorbancy values for maltose were about 27% lower and for maltotriose they were about 34% lower when estimated by the DNS method.

2.3.2 Determination of the total sugars

Total sugar in the various samples was estimated by the Phenol-Sulfuric acid method at 590 nm and the values expressed in terms of glucose equivalents (Dubois *et al.*, 1956).

2.3.3 Determination of starch

Estimation of starch in samples was carried out by using the iodine-binding method. An aliquot of 0.2 ml of sample was mixed with 0.2 ml of iodine reagent (0.1% iodine in 2% KI) and subsequent addition of 8 ml of water. The absorbance of the colour developed was recorded at 600 nm. The values were expressed as percent of the initial readings in most of cases.

2.3.4 Determination of protein

The following methods were used for the determination of protein in the enzyme samples.

2.3.4.1 Method of Lowry et al. (1951)

Protein determination in the protein purification steps was carried out using the Folin-Ciocalteau reagent as described by Lowry *et al.* (1951). Crystalline bovine serum albumin, fraction V (Sigma Chemical Company, St. Louis, USA) was used as the standard. The absrobance values were recorded at 660 nm.

2.3.4.2 Optical method

To follow the elution of protein during the various column chromatographic procedures, the absorbance measurements were carried out at 280 nm. The method was used only qualitatively.

2.3.5 Paper chromatography

Paper chromatography (Whatman filter paper no. 1) was used for the qualitative examination of the sugar composition. The solvent system used was butanol: pyridine: water (6:4:3 v/v) and the chromatograms were run in an ascending fashion for 24 h. Multiple ascents (at least two) were carried out for the better resolution of the oligosaccharides as described by Robyt and French (1963). The reducing sugars were visualized by the alkaline silver nitrate method of Trevelyan *et al.* (1950) modified by Robyt and French (1963). Hexoses and their soluble oligomers gave dark black spots.

2.3.6 Enzyme assays

The assays of various enzyme activities were performed by measuring the release of reducing sugar (RS) from the respective substrates as described in section 2.3.1. The enzymes were so diluted that O.D. differences corresponding to not more than 50 μg (Somogyi-Nelson method) or 500 μg (DNS method) were obtained. A unit (IU) of activity was defined as the amount of enzyme which produced one μ mole of glucose equivalents per min under the assay conditions described. Specific activity of the enzymes was expressed as the IU of enzyme per mg of protein.

2.3.6.1 Pullulan-hydrolysing activity

An aliquot (0.5 ml) of 2% (w/v) pullulan (Hayashibara Biochemical Company Ltd, Japan [during studies on the enzyme.production] or Sigma Chemical Company, U.S.A. [during the rest of the studies]) in 0.05 M acetate buffer, pH 4.9 was incubated with appropriately diluted enzyme (0.5 ml) for 30 min at 50°C. One unit (IU) of the enzyme activity was defined as the amount of enzyme that liberates one μmole of maltotriose (measured as glucose) from pullulan under the above experimental conditions.

2.3.6.2 α-Amylase activity

The reaction mixture contained 0.5 ml of soluble starch (2%, w/v) suspended in 0.05 M acetate buffer, pH 4.9 and 0.5 ml of suitably diluted enzyme. The reaction was carried out at 50°C for 15 min and the resulting reducing sugars produced were determined as glucose equivalents by the DNS method (section 2.3.1).

2.3.6.3 Starch-hydrolysing activity

The reaction mixture contained 0.5 ml of soluble starch (2% w/v) gelatinized at 80°C for 5 min in 0.05 M acetate buffer (pH 4.9) and suitably diluted enzyme (0.5 ml). The reaction was carried out at 50°C for 15 min and the resulting reducing sugars were determined as glucose equivalents by the DNS method (section 2.3.1).

2.3.6.4 Glucoamylase activity

The reaction mixture contained 0.5 ml of soluble starch (2% w/v) gelatinized at 80°C for 5 min in 0.05 M acetate buffer (pH 4.9) and suitably diluted enzyme (0.5 ml). The reaction mixture was incubated at 50°C for 15 min and the

reaction terminated by heating the tubes at 100°C for 5 min. Aliquots from the cooled tubes were used for the estimation of glucose by the enzymatic method. Enzymatic glucose determination kits ("Glox", Kabi Diagnostica, Sweden; "Glucoset", Ranbaxy Laboratories, India) were used as per the instructions enclosed with the respective kits.

2.4 GEL FILTRATION CHROMATOGRAPHY

A column (1.0 X 100 cm) of Bio-Gel P-150 (for purification as well as molecular weight determination) was equilibriated with 0.05 M acetate buffer (pH 4.9). Hydrated gels were throughly degassed before use. The eluate was collected in 1 - 4 ml fractions at a flow rate of 8 - 10 $ml.h^{-1}$ and assayed for protein and/or enzyme activity.

2.5 ION-EXCHANGE CHROMATOGRAPHY

A column of DEAE-Cellulose DE-52 (2 X 10 cm) was equilibriated in 0.05 M acetate buffer, pH 4.9 (column buffer). Prior to loading, the pH of the enzyme solution was adjusted to 4.9 by extensive dialysis against the column buffer. The column was washed with three bed volumes of the column buffer after loading. There after, the elution of the enzyme was carried out using a linear gradient of NaCl (0 - 0.4 M) in the same buffer. Fractions (4 ml) were collected at a flow rate of 12 - 15 $ml.h^{-1}$.

2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS

Analytical disc gel electrophoresis was performed at pH 2.9, 4.3 and 7.5 according to Maurer (1971) using different percentages of polyacrylamide gels. In case of the electrophoreses carried out at pH 2.9, uranyl nitrate was substituted for the polymerization of the gels instead of ammonium persulphate as described by

Deshpande *et al.* (1986). A current of 2.5 mA per tube was employed and the electrophoresis carried out till the marker dye (bromophenol blue or fuchsin red) had reached the bottom of the gels. Protein was visualized by staining the gels with commassie blue G-250 (Blakesley and Bozei, 1977). Carbohydrate in the duplicate gels was stained by the thymol- H_2SO_4 method (Gander, 1984).

2.7 MOLECULAR WEIGHT BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE electrophoresis containing 0.1% SDS was carried out as described by Weber and Osborn (1969) (Sigma Technical Bulletin, WS-877). The samples after pretreatment (100°C, 5 min) with 1% SDS, 1% 2-mercaptoethanol and/or 1% iodoacetamide were not dialyzed prior to electrophoresis. The electrophoresis was carried out at 6 - 8 mA per tube or 40 mA per slab gel for 8 h. The pre-stained SDS molecular weight marker kit from Sigma Chemical Company was used as per the instructions elcosed with the kit, using bromophenol blue as a tracking dye. After the electrophoresis was complete, the gels were fixed with repeated changes of 7% acetic acid to remove SDS and then stained by the method of Blakesley and Bozei (1977).

2.8 ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GEL

The procedure described by O'Farell (1975) was adopted for isoelectric focusing in polyacrylamide gels using a gel concentration of 7.5%. The pH gradient was made using 0.33 ml of Ampholines (40%, Pharmacia, pH 3.5 - 10.0) per 10 ml of the gel. Phosphoric acid (1 M) and sodium hydroxide (1 M) were used as the anode and the cathode solutions, respectively. The enzyme (60 - 100 μg) was mixed in the polymerization solutions. The focusing was carried out at 100 V for 12 h, then at 400

V for 1 h and finally at 600 V for 1 h at 4°C. After focusing, one gel was fixed in 7% acetic acid with repeated changes and then stained with commassie brillient blue R - 250 (0.2% in 7% acetic acid) overnight and destained in 7% acetic acid. One gel was cut in to pieces (2 mm), which were extracted overnight at 4°C in deionized water and then processed for estimation of pH (4°C) and enzyme activity.

2.9 PREPARATIVE ISOELECTRIC FOCUSING

Preparative isoelectric focusing was carried out in a preparative electrofocusing column (110 ml, LKB productor AB, Broma, Sweden) according to the method first described by Vesterberg and Svensson (1966). The density gradient was made either by using sucrose or glycerol using the LKB gradient mixer. Dense, light and the electrode solutions were made as per the LKB instruction manual. The enzyme sample was dialyzed overnight against glass distilled water or glycine (1%) to reduce the concentration of salts present. Ampholine carrier ampholytes (Pharmacia, pH 3.5 - 10 and 2.5 - 6.0) and the enzyme were mixed with the light and dense solutions in equal amounts before the generation of the gradient. A constant temperature circulating water-bath (LKB Multitemp 2209) was used to maintain the temperature of the column at 4°C during the focusing. Electrodes were placed such that the anode was at the top of the column. Electrofocusing was typically carried out for 48 - 72 h. Generally, the focusing run was commenced at 300 V and continued for 24 h. There after, the voltage applied to the column was periodically increased to 800 V till a stabilized current was achieved. The power applied to the column during the focusing run never exceeded 1.5 watts. After the completion of the focusing, the column was emptied at a rate of 40 - 60 $ml.h^{-1}$ and fractions of approximately 1 ml were collected.

Fractions were immediately processed for pH (4°C) and activity. The fractions showing activity were made free of glycerol or sucrose by dialysis against 0.05 M acetate buffer (pH 4.9).

2.10 DETERMINATION OF THE GLYCOPROTEIN-NATURE OF THE PURIFIED ENZYME

The glycoprotein nature of the purified pullulan-hydrolysing activity was determined by,

2.10.1 Thymol-H_2SO_4 staining technique

The glycoprotein staining method of Gander (1984) was used. The enzyme protein was electrophoresed in polyacrylamide gels (pH 7.5) and then washed at least twice in iso-propanol:acetic acid:water (25:10:65) to fix proteins and to remove low molecular weight substances. A final wash of the same fixer with 0.2% thymol (w/v) was used to stabilize the gel. The gels were then allowed to drain, suspended in a mixture of concentrated H_2SO_4 - absolute ethanol (80: 20 v/v) and shaken at 30°C. Red-brown colour of the stained glycoprotein became apparent after about 2 h. An identical gel was stained for protein by the method of Blakesley and Bozei (1977).

2.10.2 Affinity chromatography on Concanavalin A-Sepharose CL-4B

Concanavalin A forms insoluble reversible complexes with glycoproteins by reacting specifically with α-D-mannopyranosyl, α-D-glucopyranosyl residues (Goldstein *et al.*, 1965; Goldstein and Agarwal, 1968). A column (0.8 X 8.0 cm) of Concanavalin A-sepharose CL-4B was equilibriated with 0.05 M acetate buffer (pH 4.9) containing 2 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$. The glycoprotein adsorbed

on the column very strongly. It was eluted with a linear gradient of 0 - 1 M α -methyl-D-mannoside in the above buffer. The same procedure was used for enzyme purification.

CHAPTER III

OPTIMIZATION OF PULLULAN-HYDROLYSING ACTIVITY PRODUCTION IN S. rolfsii

SUMMARY

The optimization of the pullulan-hydrolysing activity production by *Sclerotium rolfsii* (NCIM 1084) was studied in shake flasks. The production of pullulan-hydrolysing activity appeared to be constitutive. Di-ammonium hydrogen orthophosphate (0.7 % w/v) was found to be the best inorganic nitrogen supplement. About 50% of the enzyme activity was produced when starch (1% w/v) was replaced at an equivalent concentration of pullulan, amylopectin, amylose, cellobiose, sucrose and glucose. The growth of the organism on lactose was found to be very poor and no enzyme activity could be detected in the culture supernatant. Supplementation of corn steep liquor (1% w/v), brought about nearly a two-fold increase in the enzyme activity (1.8 $IU.ml^{-1}$).

Effect of some additives on the thermostability of the pullulan-hydrolysing activity was also investigated. These studies were carried out at 60°C, where the half-life of the enzyme was of the order of 30 min. Out of the various sugar alcohols tested (concentrations between 0 to 5 M), xylitol and sorbitol were found to protect the enzyme activity from inactivation at 60°C. With sorbitol (more than 1.5 M) there was a complete protection of the enzyme activity for over 7 h at 60°C. Pullulan (25 $mg.ml^{-1}$) was found to provide only a limited protection against thermal inactivation in comparison with xylitol or sorbitol.

CHAPTER III

3.1 INTRODUCTION

Starch is an abundant natural reserve polysaccharide found in a number of plant species and can be efficiently utilized by a variety of microorganisms. For this, the microoganisms have developed an array of extracellular enzymes that can attack and depolymerize starch to glucose and higher maltodextrins. The extracellular enzymes that catalyze the depolymerization of starch are α-amylase (EC 3.2.1.1), β-amylase (EC3.2.1.2) and glucoamylase or γ-amylase (EC3.2.1.3), which hydrolyze the α-(1->4)-linkages in amylose and amylopectin, the constituent polymers of starch. On the other hand, pullulanase (debranching enzyme, EC 3.2.1.41) selectively attacks the α-(1->6)-glucosidic branch points in amylopectin and accelerates the complete hydrolysis of starch (Fogarty, 1983; Kennedy *et al.*, 1988). In fact, by choosing a proper combination and the concentration of these enzymes, one can get a desired end-product with a defined concentration of glucose and maltooligosaccharides (Kennedy *et al.*, 1988).

From the commercial point of view, the majority of the α-amylases used in the liquefaction of starch are from bacterial sources such as, *Bacillus amyloliquefaciens*, *B. licheniformis* etc. The saccharification of the thinned out starch slurry is carried out by the use of fungal glucoamylases which results in the formation of high glucose "syrups". The industrially used glucoamylases are from *Aspergillus awamori*, *Aspergillus oryzae*, and *Rhizopus* species. Pullulanases applied in the industrial processing of starch are of bacterial origin. These are either from *Klebsiella oxytoca* (formerly *K. pneumoniae*, *Aerobacter aerogenes*) and *Bacillus acidopullulyticus* (Promozym 200L from Novo Nordisk Biolabs, Denmark).

The literature survey shows that there are only a few reports, wherein fungal amylolytic enzymes have been reported to act on pullulan (Saha *et al.*, 1979; McCleary and Anderson, 1980; DeMot and Verachtert, 1987). Sakano *et al.* (1972) had screened fungal strains for pullulan-hydrolysing activity and reported pullulan 4-glucanohydrolase (EC 3.2.1.57) present in *Aspergillus niger*. This enzyme was found to act on the α-(1->4)-linkages in pullulan producing isopanose. We had found that *Sclerotium rolfsii* (NCIM 1084) had a potential for the production of pullulan-hydrolysing enzymes. Hence, the present investigations were undertaken to optimize conditions to increase the yield of the pullulan-hydrolysing activity.

Results pertaining to the optimization of the medium for the extracellular production of pullulan-hydrolysing activity, which are presented in this chapter, have been published (Kelkar *et al.*, 1988).

3.2 CULTURE CONDITIONS FOR THE MAXIMIZATION OF PULLULAN-HYDROLYSING ACTIVITY PRODUCTION

3.2.1 Results

An increase in the extracellular production of pullulan-hydrolysing enzyme(s) was obtained by optimizing the nutrients provided in culture. All the experiments reported have been repeated at least twice with similar results.

3.2.1.1 Influence of inorganic nitrogen sources

Submerged fermentations of *S. rolfsii*, were carried out in shake flasks at 28 - 30°C in NM-2 medium (Sadana *et al.*, 1979) with hydrolysed starch (1% w/v) as sole carbon source for 10 days.

The influence of the various inorganic nitrogen sources on the production of pullulan-hydrolysing activity is tabulated in Table 3.1. Though all of the nitrogen salts tested supported good growth of the organism, none of the salts could effectively replace $(NH_4)_2HPO_4$ as the inorganic nitrogen source in the NM-2 medium. Therefore, in the subsequent studies, di-ammonium hydrogen orthophosphate (0.7 % w/v) was used as the inorganic nitrogen source.

3.2.1.2 Effect of carbon source

Various carbon sources in the form of monosaccharides, disaccharides, trisaccharides and polysaccharides were added to the basal medium omitting starch and their effect on the enzyme production was studied (Table 3.2).

Significant levels of pullulan-hydrolysing activity were observed to be produced not only with starch (0.67-1.06 $IU.ml^{-1}$), but also with pullulan (0.18-0.70 $IU.ml^{-1}$), amylopectin (0.6-0.7 $IU.ml^{-1}$) and glucose (0.62-0.79 $IU.ml^{-1}$) at 1% (w/v) level during 5-10 days of cultivation. Growth and enzyme activities were lower with glycerol, rhamnose, gentiobiose and raffinose (0.05-0.35 $IU.ml^{-1}$) during 5-10 days of cultivation. In presence of lactose growth was poor and no enzyme activity was detectable.

3.2.1.3 Effect of starch concentration

Effects of different concentrations of the hydrolysed starch was observed to check its influence on the production of pullulan-hydrolysing activity (Table 3.3). It was found that the levels of the pullulan-hydrolysing activity showed an increase of about 1.5-fold when hydrolysed starch at a concentration of 3% (w/v) was used in the medium. Further increase in the concentration of starch (to 4%) resulted in a nearly 60% loss in the enzyme activity as compared to the levels observed with 1% of starch.

Table 3.1: Influence of various inorganic nitrogen sources on the production of extracellular pullulan-hydrolysing activity by *S. rolfsii*.

Inorganic Nitrogen Source	Day 5		Day 10	
	pH	IU.ml^{-1}	pH	IU.ml^{-1}
$(NH_4)_2SO_4$, 0.14%[a]	**2.5**	**0.13**	**2.4**	0.31
$(NH_4)_2)SO_4$,0.28-0.7%	2.6	**0.25**	**2.6**	0.42
NH_4NO_3, 0.18%[a]	**3.9**	**0.06**	**3.1**	0.21
KNO_3, 0.23%[a]	**4.0**	**0.09**	**2.8**	0.32
$(NH_4)_2HPO_4$				
0.14%	**2.5**	0.16	**2.5**	0.48
0.24-0.48%	**2.7**	0.26	**2.6**	0.7
0.7%	**3.1**	0.67	2.9	1.05

Note : Cultures were grown on $(NH_4)_2HPO_4$-free NM-2 medium plus inorganic nitrogen source indicated, with 1% (w/v) starch

a Nitrogen source added at equivalent nitrogen levels.

Table 3.2: Effect of different carbon sources on the production of extracellular pullulan-hydrolysing activity by *S. rolfsii*.

Carbon source[a]	Day 5		Day 10	
	pH	IU.ml^{-1}	pH	IU.ml^{-1}
Glycerol	3.8	0.06	3.7	0.25
Glucose	3.0	0.62	2.7	0.79
Mannose	2.9	0.16	3.3	0.38
Rhamnose (0.5 %)	4.8	0.05	3.6	0.27
Maltose	3.0	0.37	3.1	0.42
Sucrose	3.0	0.37	2.9	0.65
Lactose	6.3	-	6.0	-
Cellobiose	3.3	0,23	3.0	0.68
Gentiobiose	3.3	0.14	3.1	0.29
Melibiose	5.5	0.07	3.5	0.50
Raffinose	4.9	0.08	3.3	0.35
Amylose	3.1	0.64	3.3	0.59
Amylopectin	3.1	0.60	3.2	0.70
Pullulan	3.1	0.18	2.5	0.70
Starch	3.1	0.67	3.0	1.06

Note : Cultures were grown on starch-free NM-2 medium plus the carbon source indicated

a Carbon source concentration was 1% (w/v), unless otherwise indicated

Table 3.3 : Effect of different concentrations of starch on the production of extracellular pullulan hydrolysing activity by *S. rolfsii*.

	Day 5		Day 10	
Soluble starch (w/v)[a]	pH	IU.ml^{-1}	pH	IU.ml^{-1}
1%	**3.1**	0.67	**3.0**	1.06
2%	**3.1**	0.72	**2.8**	1.35
3%	**3.0**	0.78	**2.6**	1.62
4%	**2.9**	0.70	**2.5**	0.40

a Starch concentration in the NM-2 medium

3.2.1.4 Effect of surface active agents

The addition of surfactants has been reported to increase the extracellular enzyme production in microorganisms (Reese *et al.*, 1969, Panda *et al.*, 1987). The effect of surfactant mediated increase in the extracellular activity has been attributed to:

1. their action on the cell membrane causing an increase in permeability (Reese *et al.*, 1969)
2. partly to the formation of inducers and partly to the promotion of the release of the cell-bound enzyme (Ksandopulo, 1974; Lisker *et al.*, 1975; Gomez-Alarcon *et al.*, 1989)
3. release of metabolizable fatty acids from Tween which promote growth (Asther *et al.*, 1987; Panda *et al.*, 1987)

Effect of addition of various surfactants to Tween-80 free NM-2 medium on the enzyme production is summarized in Table 3.4. Tween-80 (0.033% v/v) gave the highest enzyme activity. Tween-20 and Triton-X-100 (both at 0.033% v/v) inhibited growth to a large extent affecting the enzyme production. Enzyme production with the addition of Alkaterge-E and Span-20 (0.033% v/v) was between (0.15-0.30 $IU.ml^{-1}$) but lowered to 0.10 $IU.ml^{-1}$ on the 10th day of cultivation.

3.2.1.5 Influence of organic supplements

Effect of addition of various organic supplements (1% w/v) to the NM-2 medium on the pullulan-hydrolysing activity is given in Table 3.5. Addition of corn steep liquor, wheat bran, cotton seed meal and coconut meal increased yield of the enzyme significantly (40-80%) as compared to the control. Enzyme yield with the supplementation of soya meal, oat meal, ground nut cake or rice bran was lower

Table 3.4 : Effect of different surfactants on the extracellular production of pullulan-hydrolysing activity by *S. rolfsii*.

Surfactant (v/v)[a]	Day 5		Day 10	
	pH	IU.ml^{-1}	pH	IU.ml^{-1}
None	3.4	0.03	4.0	0.13
Tween-80				
0.1%	4.3	0.06	3.3	0.16
0.05%	4.2	0.18	3.3	0.21
0.033%	3.1	0.60	2.9	1.00
Tween-20				
0.033%	6.3	-	6.1	-
Triton X-100				
0.033%	6.1	0.09	5.5	0.12
Span-20				
0.033%	4.2	0.30	3.8	0.10
Alkaterge-E				
0.033%	4.1	0.15	3.4	0.11

a Note : Cultures were grown in Tween-80 free NM-2 medium with the surfactants at the indicated concentrations

Table 3.5 : Effect of organic additives on the extracellular production of pullulan-hydrolysing activity by *S. rolfsii*.

Organic additive[a]	Day 5		Day 10	
	pH	$IU.ml^{-1}$	pH	$IU.ml^{-1}$
None	**3.1**	0.67	2.9	1.05
Corn steep liquor	**3.1**	1.00	3.2	1.80
Wheat bran	**2.6**	0.63	3.0	1.40
Rice bran	**2.8**	0.30	3.0	0.90
Cotton seed meal	**2.8**	0.80	**3.0**	1.60
Oat meal	**2.7**	0.54	3.0	1.20
Groundnut Cake	**3.2**	1.00	3.1	1.20
Soya meal	**2.8**	1.00	3.0	1.20
Coconut meal	**2.7**	1.20	2.9	1.50
Yeast extract				
0.01%	**3.3**	0.70	3.0	0.95
0.02%	3.1	0.90	3.1	0.72

Note : Cultures grown on NM-2 medium with the organic additive as indicated.

a Organic additive concentration was 1% (w/v), unless otherwise mentioned.

(0.30-1.35 IU.ml^{-1}) during 5-10 days of cultivation. A maximum increase of about 1.8-2.0 fold was observed with corn steep liquor (1% w/v) on the 10th day of fermentation.

3.2.1.6 Effect of initial pH of the medium

Studies on the effect of initial pH (before autoclaving) of the medium on the production of pullulan-hydrolysing activity were carried out within the pH range of 3.0 - 7.5 (Table 3.6). An initial pH of between 6.0 - 6.5 was found to be optimum for the production of the enzyme activity. Growth, as well as, enzyme production were observed to be affected at pH lower than 5.0 and higher than 7.0. *S. rolfsii* grows within the pH range indicated and the pH of the culture medium drops rapidly by 5th day subsequent to the accumulation of formic acid and oxalic acid in the medium (Takao *et al.*, 1986).

3.2.1.7 Formation of pullulan-hydrolysing activity during the growth cycle of S. rolfsii

The course of production of pullulan-hydrolysing activity and the pH profile are shown in Fig. 3.1. During the exponential phase of growth, the pH of the culture medium was observed to fall rapidly to around 3.0 (4 days). Detectable levels of the pullulan-hydrolysing activity were observed in the culture supernatant from the 3rd day of cultivation. The enzyme activity showed an increase until 10th day even when the starch present in the supernatant had disappeared (as determined from the iodine binding characteristics) by the 5th day of cultivation. If the cultivation was continued further, there was no increase observed in the activity. On the contrary, the levels of the enzyme activity dropped slightly.

Table 3.6 : Effect of initial pH of the medium on the extracellular production of pullulan-hydrolysing activity by *S. rolfsii*.

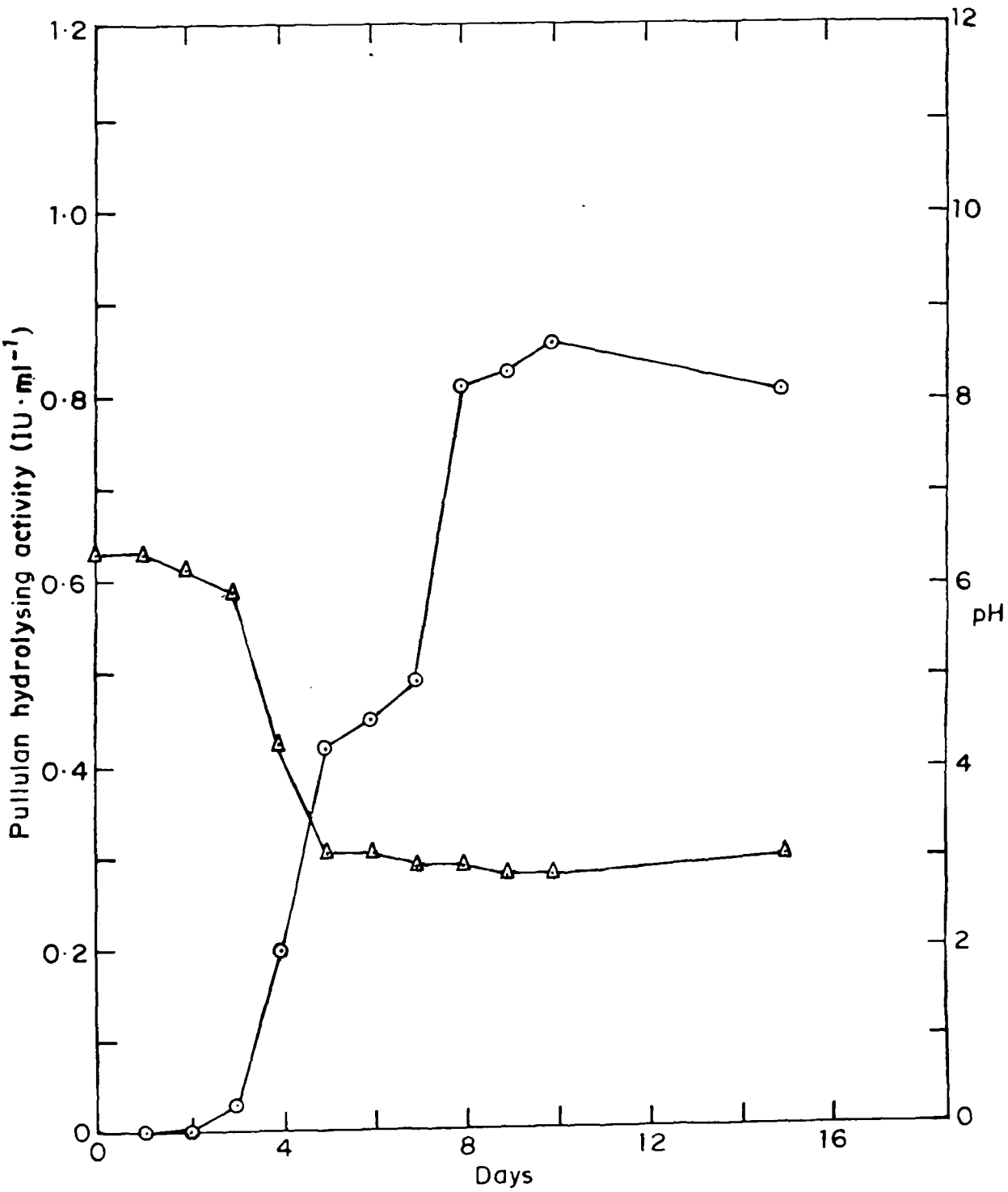
Initial pH[a]	Day 5		Day 10	
	pH	IU.ml^{-1}	pH	IU.ml^{-1}
3.0	2.7	0.31	2.6	0.40
3.5	2.7	0.36	2.7	0.67
4.0	2.8	0.36	2.7	0.67
5.0	2.8	0.36	2.8	0.67
5.5	2.8	0.72	2.8	0.99
6.0	2.8	0.61	2.8	1.02
6.5	3.0	0.65	2.8	1.05
7.0	3.9	0.35	2.8	0.78
7.5	6.8	-	6.7	-

a Initial pH of the NM-2 medium adjusted to the respective pH values indicated.

Fig. 3.1: pH profile and production of extracellular pullulan-hydrolysing activity in NM-2 medium during a 15-day cultivation of *S. rolfsii*.

(▲) pH profile

(⊙) Production of pullulan-hydrolysing activity


1·2
1·0
0·8
0·6
0·4
0·2
0
Pullulan hydrolysing activity (IU·ml⁻¹)
12
10
8
6
4
2
0
pH
0
4
8
12
16
Days

3.2.1.8 Biochemical characterization of the native pullulan-hydrolysing enzyme(s)

Some biochemical properties of the native enzyme were examined using the culture filtrate (10 d of fermentation) dialyzed against 0.05 M citrate buffer, pH 4.2 (4°C, 16 h).

The enzyme was stable over a broad temperature range of 25 - 55°C (pH 4.2, 30 min). A 100% loss in activity was observed at 70°C under identical conditions. Optimum temperature for the activity was 50°C and the E_a (activation energy), calculated from the Arrhenius plot, was 22.6 $kJ.mol^{-1}$. The enzyme was active in the pH range of 3.0 - 6.8; with an optimum at 4.2. Under the experimental conditions (50°C, 30 min), the enzyme activity was 70 - 95% stable in the pH range of 3.5 - 5.5. Using pullulan as a substrate, under standard assay conditions, the apparent K_m was calculated to be 8.33 $mg.ml^{-1}$ by the Lineweaver-Burk plot, whereas V_{max} (μmol glucose equivalents.min^{-1}.ml of culture filtrate^{-1}) was 2.2.

3.2.2 Discussion

An increase in the production of pullulan-hydrolysing activity by *S. rolfsii* (N.C.I.M. 1084) was obtained by the manipulation of the nutrient medium constituents. There was no effective replacement for diammonium hydrogen orthophosphate (0.7 % w/v) as an inorganic nitrogen source in the medium. None of the other nitrogen salts tested were capable of effectively replacing it for the optimum production of pullulan-hydrolysing activity in culture.

The carbon source provided in the medium affects the type and the amount of enzyme activities produced in the extracellular environment by the microorganisms. Since, soluble starch was found to be capable of supporting the production of

pullulan-hydrolysing activity in *S. rolfsii*, a number of other α-D-glucans and their constituent monosaccharides were tested for their ability to induce or repress the production of pullulan-hydrolysing activity. In literature, there are contrasting reports regarding the ability of various mono- and poly-saccharides to induce the synthesis of the pullulan-hydrolysing enzymes. In case of *K. oxytoca*, it is known that the pullulanase activity is encoded by the "*pul C*" operon that comes under the coordinate control of the *Mal T* regulon and is induced by the presence of maltose (Pugsley and Reyess, 1990). Also, in case of *Clostridium thermohydrosulfuricum* the synthesis of pullulanase and glucoamylase was shown to be induced only in the presence of maltose and other carbohydrates containing maltose units (Hyun and Zeikus, 1985). Similar results were also reported in the case of *Clostridium* strain EM 1 (Madi *et al.*, 1987) and *C. thermohydrosulfuricum* E 101-69 (Melasniemi, 1987).

In case of *S. rolfsii*, it was observed that almost all of the α-D-glucans tested were capable of supporting the extracellular production of pullulan-hydrolysing activity. The most notable results were still obtained with hydrolysed starch as the carbon source (1.05 $IU.ml^{-1}$). Similar observations were made by Kimura and Horikoshi (1989), who found that the synthesis of pullulanase in the alkalopsychrotrophic *Micrococcus* sp. 207 was induced most effectively by soluble starch and dextrins. In case of the pullulanase from *Bacillus stearothermophilus* KP 1064, Suzuki and Chishiro (1983) noted that starch and dextrin (0.172 $U.ml^{-1}$ and 0.175 $U.ml^{-1}$, respectively) in the medium were the best inducers of the pullulanase activity. Surprisingly, though pullulan was used as a substrate for growth, it did not support extracellular production of the enzyme activity (0.006 $U.ml^{-1}$), in case of *B. stearothermophilus* KP 1064 (Suzuki and Chishiro, 1983). Odibo and Obi (1990) have also observed that the production of pullulanase in case of *Thermoactinomyces*

thalpophilus no. 15, was greatly stimulated by the use of various starches (yam, cassava, rice etc.) as a carbon source, with levels of activity ranging from 27 to 73 $U.ml^{-1}$. In comparison, pullulan repressed the synthesis of pullulanase (9 $U.ml^{-1}$) though the growth of the organism was found to be unaffected.

S. rolfsii when growing on glucose as the sole carbon source was found to be capable of producing the pullulan-hydrolysing activity (0.6 - 0.8 $IU.ml^{-1}$). This observation apparently indicates that the synthesis of pullulan-hydrolysing activity in *S. rolfsii* is constitutive and is not repressed by presence of glucose in the culture medium. These observations are in contrast to those made with *C. thermohydrosulfuricum* E 101-69 (Melasniemi, 1987) and *C. thermohydrosulfuricum* (Hyun and Zeikus, 1985), who found that glucose severely repressed the synthesis of pullulanase in these organisms. Addition of mannose, xylose, fructose, cellobiose and lactose was found to completely repress the production of pullulanase in *C. thermohydrosulfuricum* (Hyun and Zeikus, 1985). Glucose was also reported to repress the synthesis of pullulanase in *Micrococcus* sp. 207 (Kimura and Horikoshi, 1989).

Though there was an increase in the enzyme activity observed with starch (3%), for the rest of the study, use of starch at a concentration of 1% was preferred. This had to be done because of the fact that the higher concentrations of starch supported a very luxuriant growth of *S. rolfsii* which was accompanied by production of an excess of exo-polysaccharide ("scleroglucan"). This resulted in a very viscous fermentation broth that became difficult to handle. This observation was consistent with the reports of use of concentrated starch suspensions for the industrial production of scleroglucan (Ferguson and Westover, 1969).

Inclusion of Tween-80 at a concentration of (0.033% v/v) gave a significant increase (7-fold) in the enzyme activity as compared to the control in case of *S. rolfsii*. Similar observations were made by Deshpande (1981) for the production of cellulases by *S. rolfsii*. Ksandopulo (1974) and Asther *et al.* (1987) have also reported that the use of Tween's of different specifications in the culture medium enhanced the production of lipase from *Geotrichum* spp. and ligninase from *Phanerochaete chrysosporium* INA-12, respectively. It was postulated that the lipolytic activity produced by the fungus breaks down the Tween's, liberating free fatty acids that are metabolized by *P. chrysosporium* INA-12. These findings were confirmed by the addition of exogenous oleic acid, which was found to markedly improve the ligninase production in *P. chrysosporium* INA-12 (Asther *et al.*, 1987).

But these findings can not be considered as a comprehensive proof for the changes in fatty acid metabolism in case of these fungi. Jaeger *et al.* (1985) have found that the addition of a detergent 3- (3-cholamidopropyl)dimethylammonio -1-propanesulfonate (CHAPS) affords a comparable ligninase activity in case of *P. chrysosporium* cultures when compared to those supplemented with Tween-80. CHAPS in fact does not contain any fatty acids but produces the same effect as Tween-80 does.

Panda *et al.* (1987) have studied the stimulation of protein secretion in case of *T. reesei* by various Tween's. They have concluded from their studies that the surfactant did not appear to be exerting its effect via release of the enzyme from the cell surface nor through the changes in the growth rate and the overall fatty acid composition of the fungus. It was known that low producer strains of *Trichoderma* (as far as the cellulases are concerned) were limited in their secretory capacity at the level of the membrane biogenesis, which could be relieved by the supplementation of lipid

precursors in the medium (Ghosh *et al.*, 1984). Thus, it was speculated that the findings of increased lipid contents in mycelia of *T. reesei* supplemented with Tween-60 and Tween-80 could be in accordance with this observation (Panda *et al.*, 1987). More work is needed to elucidate the reason behind the enhancing effect of addition of Tween-80 on the production of pullulan-hydrolysing activity by *S. rolfsii*.

Inclusion of different organic supplements in the culture medium is known to influence the production of extracellular pullulanase production in microorganisms (Takasaki, 1976a; Suzuki and Chishiro, 1983; Odibo and Obi, 1990). The organic supplements not only provide micro-nutrients but can potentially supply additional carbon and nitrogen in the medium. Addition of various oil seed meals in the medium, a by-product from the oil industry, was found to improve the yield of pullulan-hydrolysing activity significantly (Table 3.5). The most significant effect was observed in the case of corn steep liquor which gave nearly a 2-fold increase in the enzyme activity observed on the 10th day of fermentation. One significant observation was the early achievement of a comparable level of enzyme activity (5th day of fermentation), which was normally obtained in unsupplemented control only on the 10th day of fermentation. This can potentially cut down the time of fermentation to half when compared with the unsupplemented control.

Odibo and Obi (1990) working with *Thermoactinomyces thalpophilus* no. 15 found that the actinomycete preferred organic nitrogen supplements to inorganic ones. They found that addition of soybean meal or cotton seed meal in the medium gave significantly better activity of pullulanase (11 - 15 fold more) when compared with the inorganic nitrogen source alone, i.e. ammonium sulfate. A similar requirement for the organic nitrogen sources has been reported in *Bacillus* sp. (Takasaki, 1976a; Suzuki and Chishiro, 1983) for the production of pullulanase.

3.3 INFLUENCE OF VARIOUS ADDITIVES ON THE THERMOSTABILITY OF PULLULAN-HYDROLYSING ACTIVITY

In case of industrial enzymatic processes, increasing the temperature of the reaction often results in an accelaration of the rate of reaction. But after a certain point it starts resulting in the thermal inactivation of the enzyme catalyst. This at times becomes the restricting factor in practical application of some processes. Stabilizing the enzyme catalyst against thermal denaturation therefore becomes important.

A study was undertaken to assess the effects of various additives on the thermostability of the pullulan-hydrolysing activity in *S. rolfsii*.

3.3.1 Results

3.3.1.1 Determination of the half-life of pullulan-hydrolysing activity at different temperatures

The half-life of the enzyme was studied by periodically sampling the enzyme kept at different temperatures (0.05 M acetate buffer, pH 4.9) and then determining the residual enzyme activity as described in the section 2.3.1.1 (Table 3.7). A temperature of 60°C was chosen as the inactivation temperature for studying the thermostability of the pullulan-hydrolysing activity. The half-life of the enzyme obtained at this temperature was short enough (~ 30 min) for sufficient precision and to allow the experiments to be performed rapidly.

Table 3.7 : Half-life of the pullulan-hydrolysing activity from *S. rolfsii* at different temperatures.

Temperature (°C)	Half-life (min)
50	more than 420
55	210
60	30
65	13
70	4

3.3.1.2 Effect of polyhydric alcohols on the thermostability of pullulan-hydrolysing activity

The stabilizing effect of different polyhydric alcohols containing two to six carbon atoms was studied (Fig. 3.2 a - f). The polyhydric alcohols used included ethylene glycol, glycerol, xylitol and sorbitol. The results presented in Fig. 3.3, show an increase in the protective effect of polyols with an increase in the length of the carbon chain and the number of OH groups present per molecule. Thus to achieve a similar effect glycerol (three OH per molecule) has to be used at a much higher concentration (4 M) as compared to sorbitol (six OH per molecule), which can be used at concentration of 0.5 M. Sorbitol and xylitol had a marked effect on the thermostability of the enzyme activity (Fig. 3.2 a - f). The most noticeable effects in case of these sugar alcohols were found at a concentration higher than 1.5 M. There was a complete and continued protection of the enzyme activity up to 7 h when sorbitol was used at a concentration of more than 1.5 M. In contrast, with xylitol at a concentration of more than 3 M it was possible to achieve the same effect as that of sorbitol. Short chain alcohols tested (ethylene glycol and glycerol) were not found to be as effective as sorbitol and xylitol (Fig. 3.2 a - f). In case of ethylene glycol, the enzyme activity was observed to be rapidly inactivated as compared to the control without any additives.

3.3.1.3 Effect of addition of substrate on the thermostability of pullulan-hydrolysing activity

It has been shown that the thermostability of the enzymes may be enhanced by inclusion of their respective substrates in the reaction mixture (Citri, 1973). It was seen that by including pullulan at two concentrations (5 and 25 $mg.ml^{-1}$) contributed

Fig. 3.2 a: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(●) Control
(•——•) Ethane-diol
(△——△) Glycerol
(○——○) Xylitol
(×——×) Sorbitol

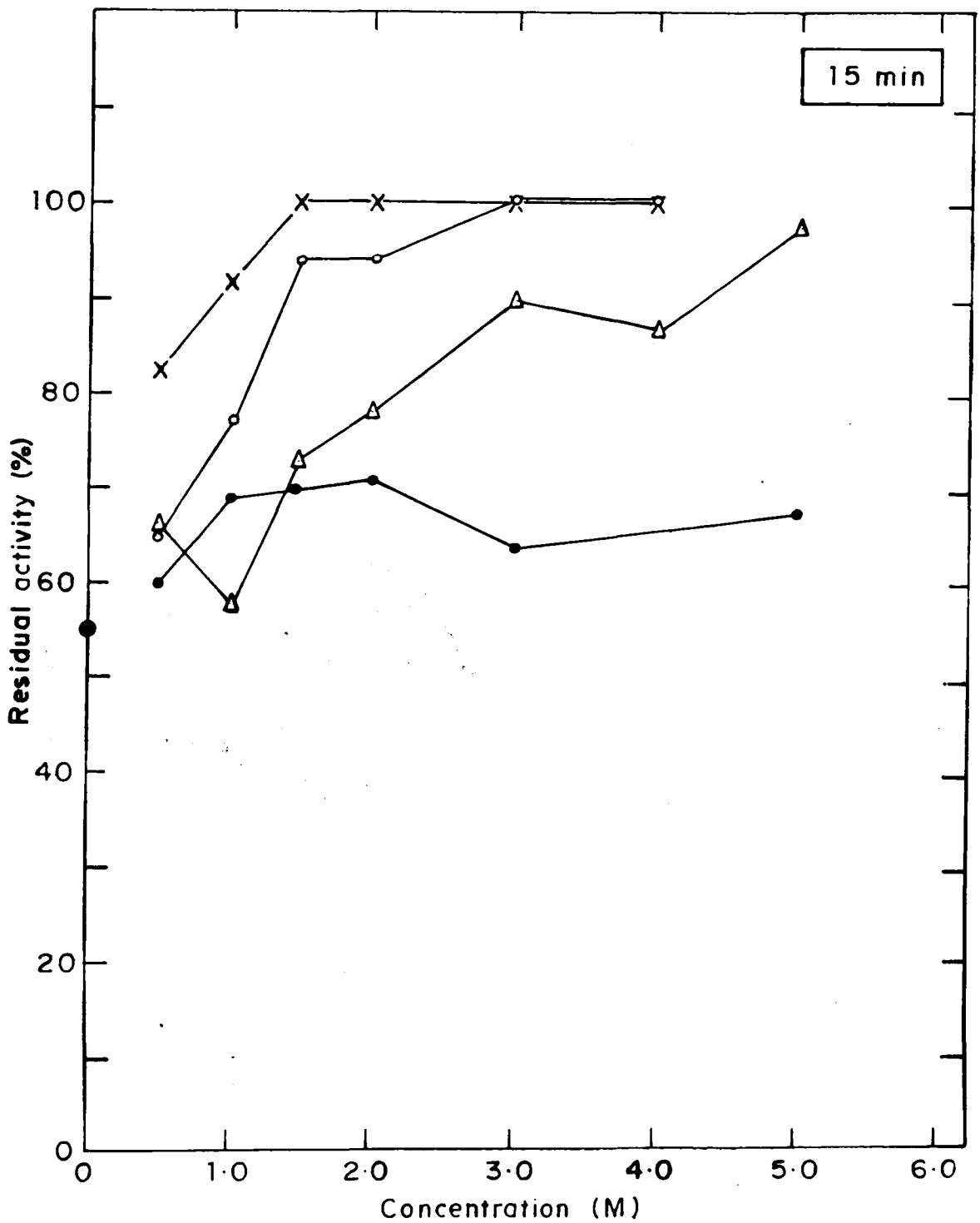

15 min
100
80
60
40
20
0
Residual activity (%)
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.2 b: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(●) Control
(•——•) Ethane-diol
(△——△) Glycerol
(○——○) Xylitol
(×——×) Sorbitol

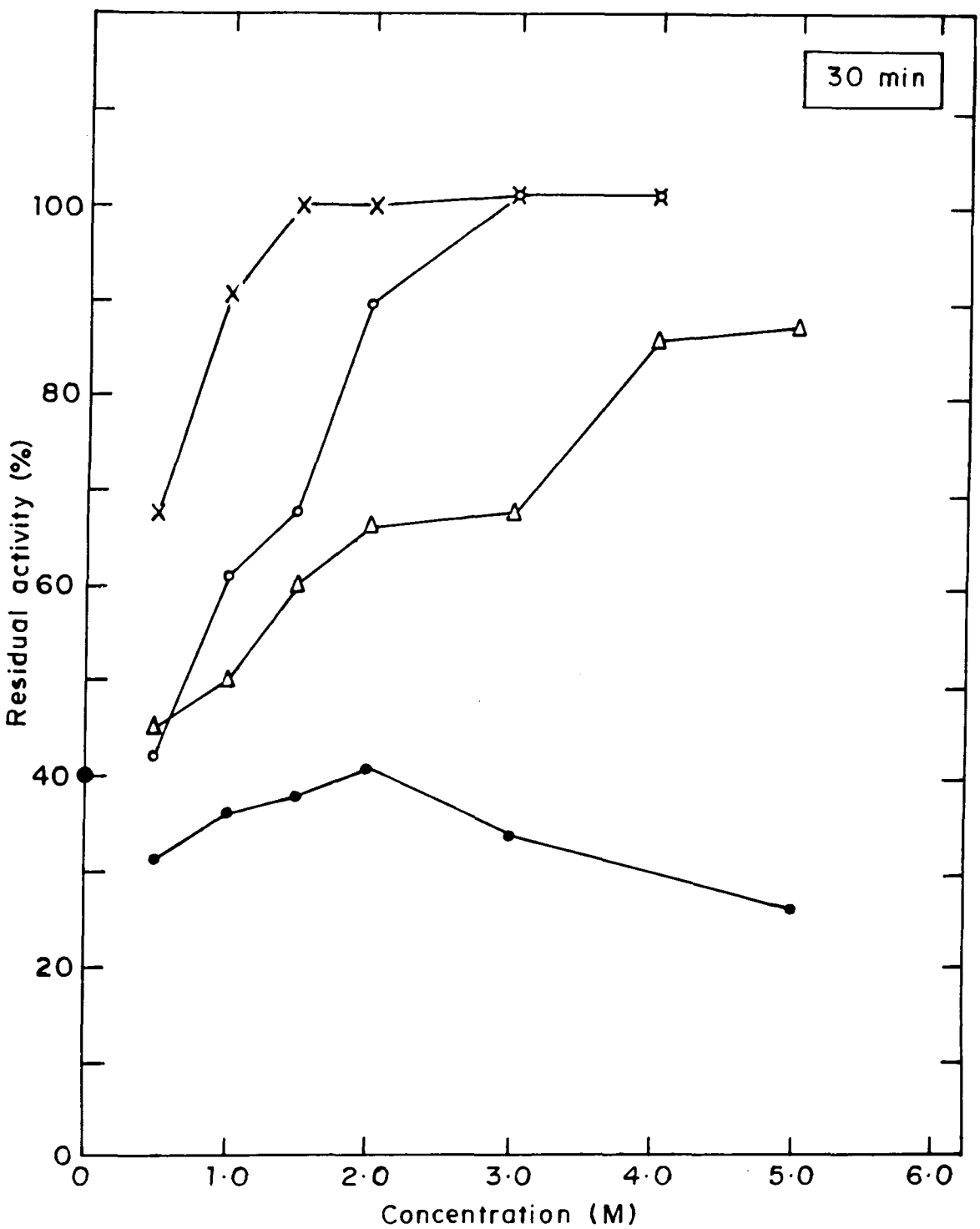
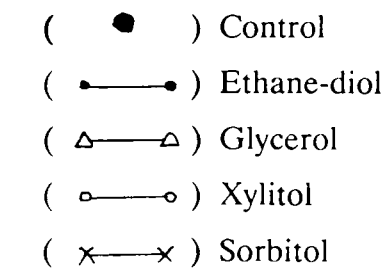

30 min
Residual activity (%)
0
20
40
60
80
100
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.2 c: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.



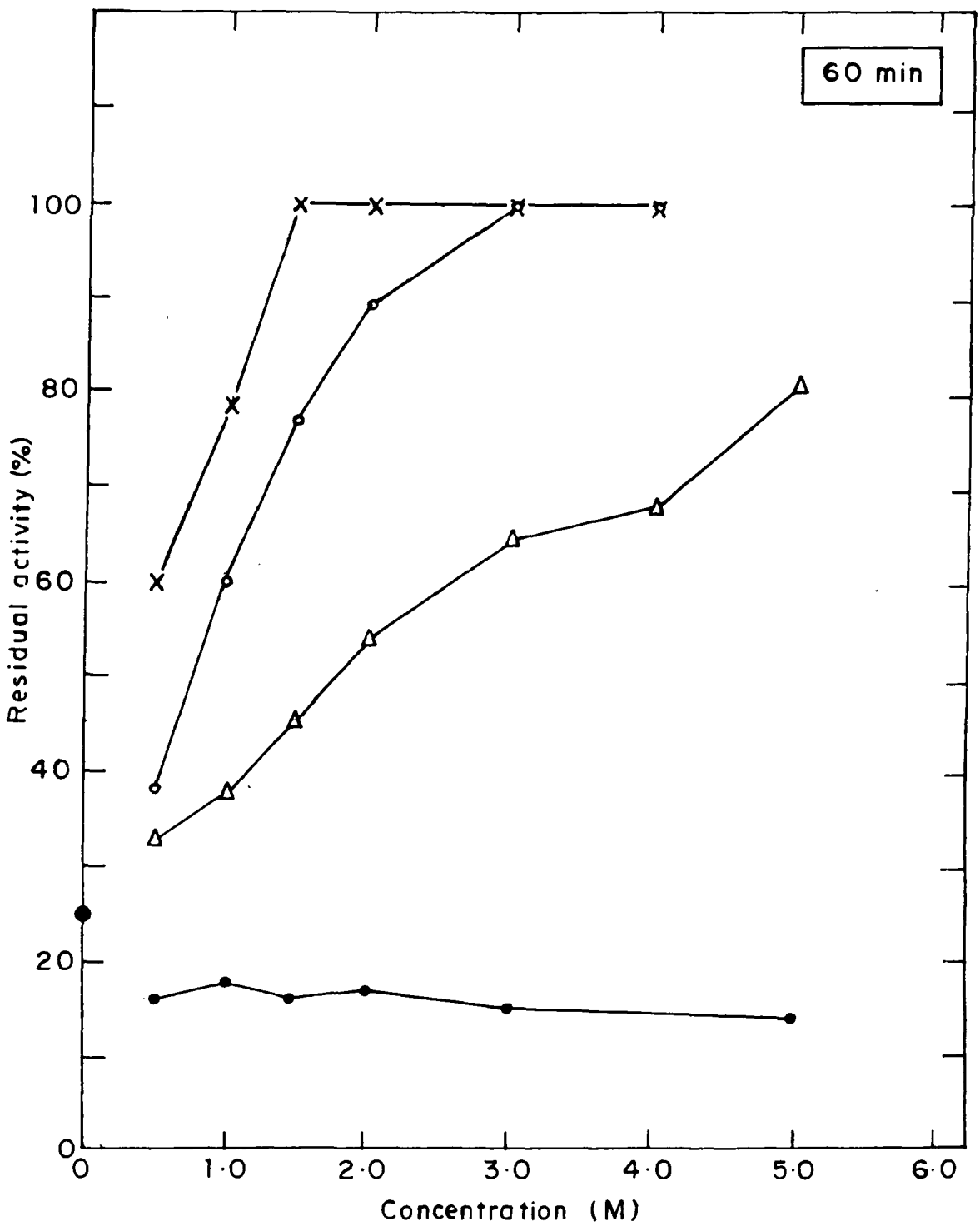

60 min
100
80
60
40
20
0
Residual activity (%)
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.2 d: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(●) Control
(●——●) Ethane-diol
(△——△) Glycerol
(○——○) Xylitol
(×——×) Sorbitol

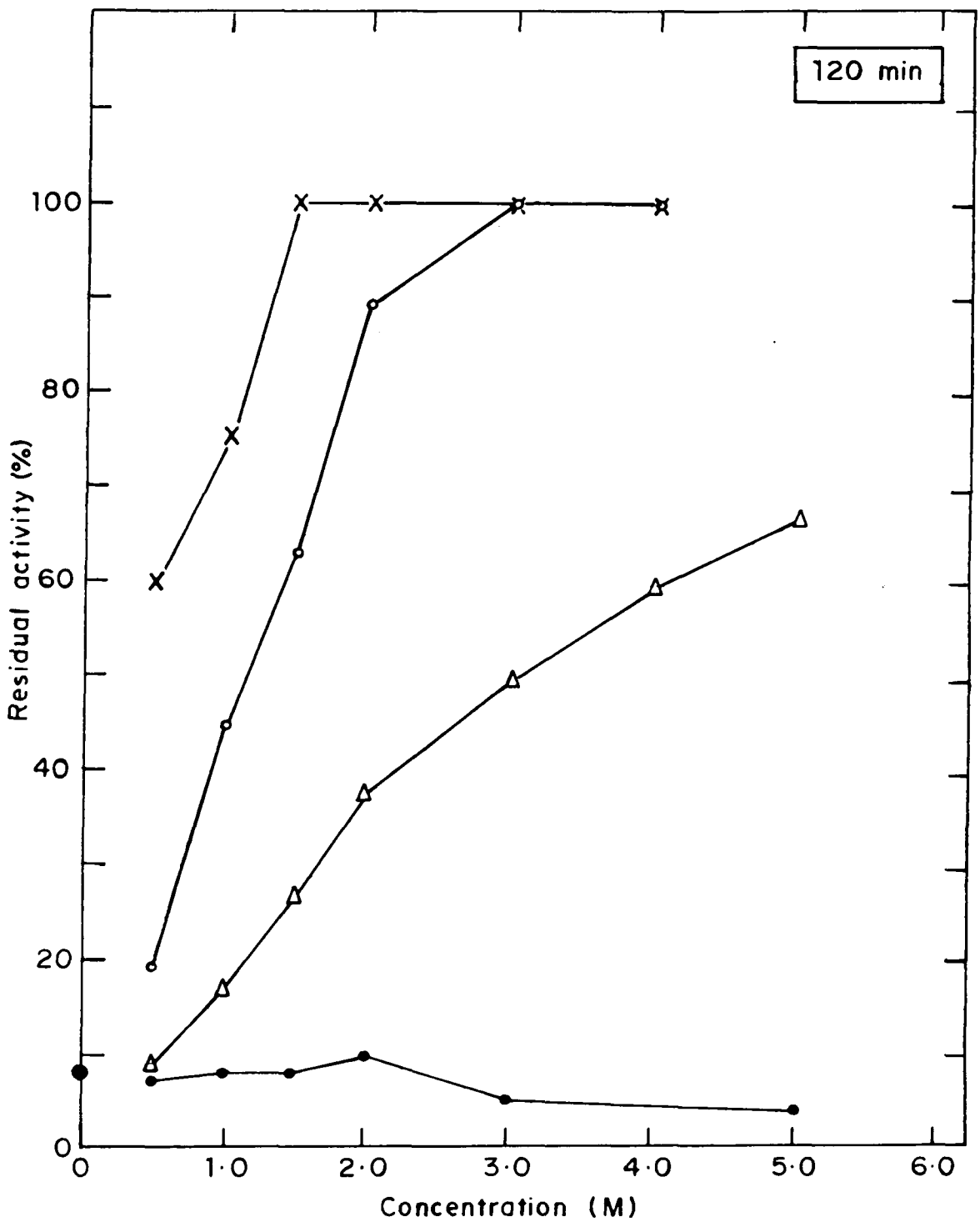

120 min
Residual activity (%)
0
20
40
60
80
100
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.2 e: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(●) Control
(●——●) Ethane-diol
(△——△) Glycerol
(○——○) Xylitol
(×——×) Sorbitol

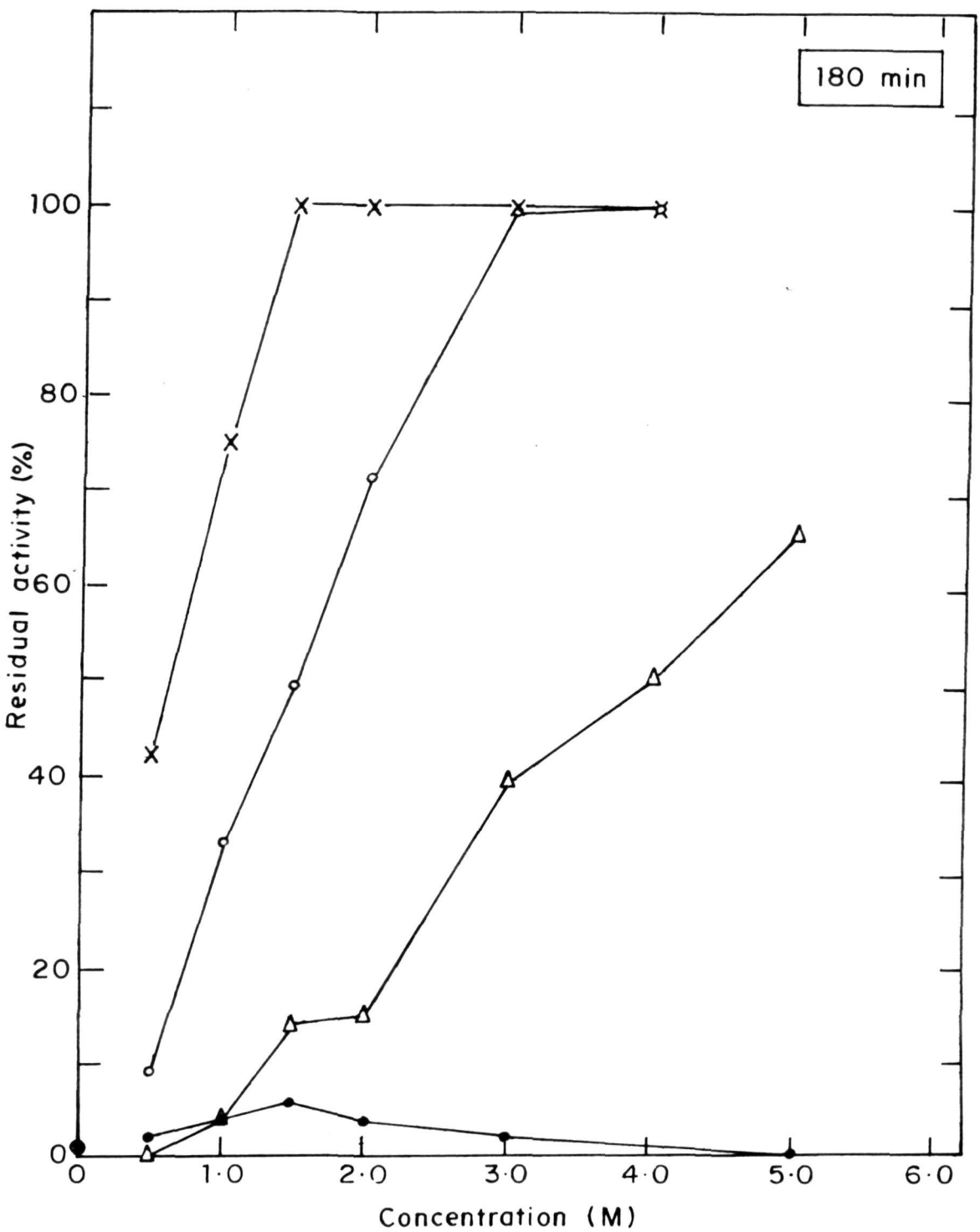

180 min
Residual activity (%)
100
80
60
40
20
0
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.2 f: Effect of polyhydric alcohol concentration on the pullulan-hydrolysing activity . Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(●) Control
(•——•) Ethane-diol
(△——△) Glycerol
(○——○) Xylitol
(×——×) Sorbitol

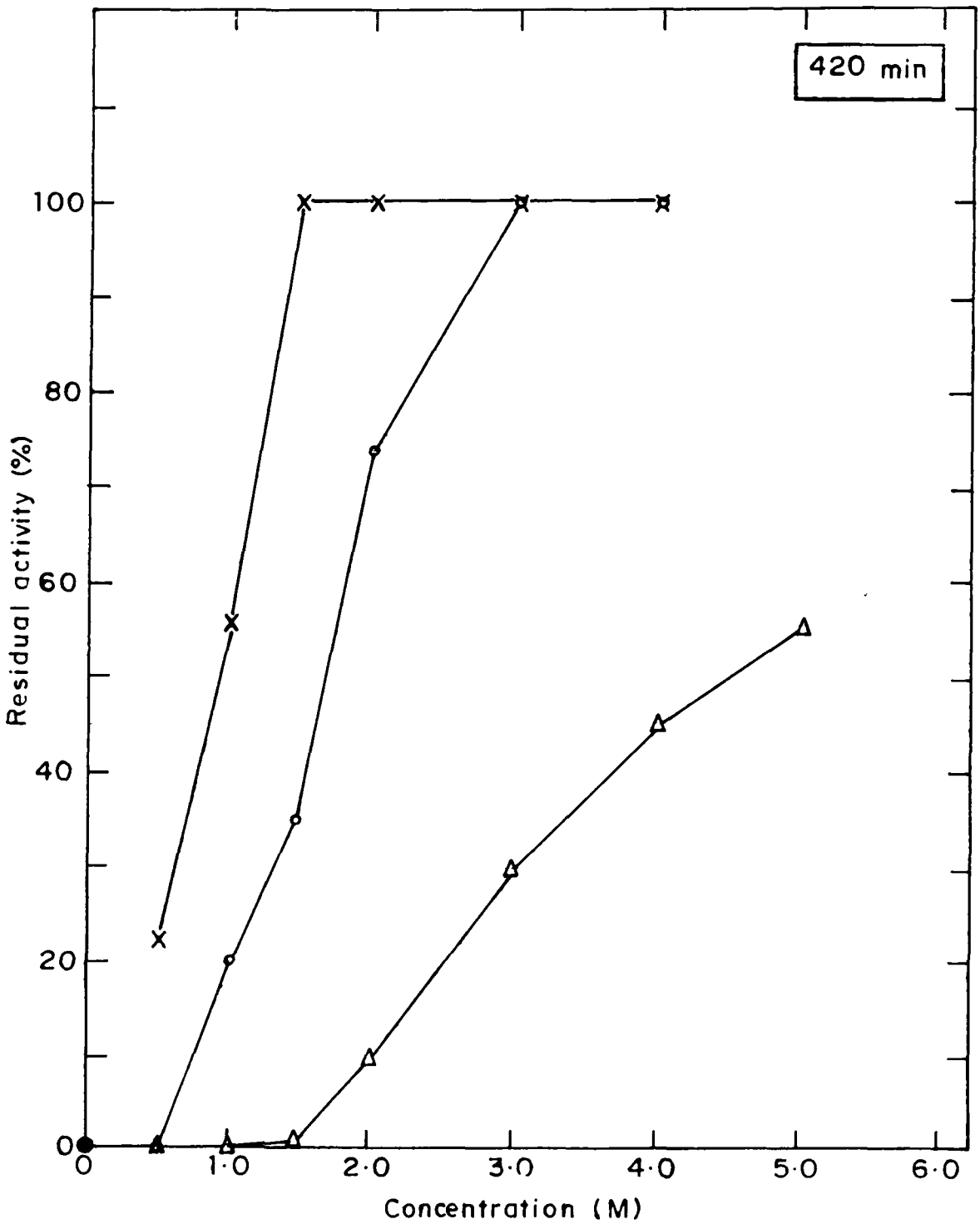
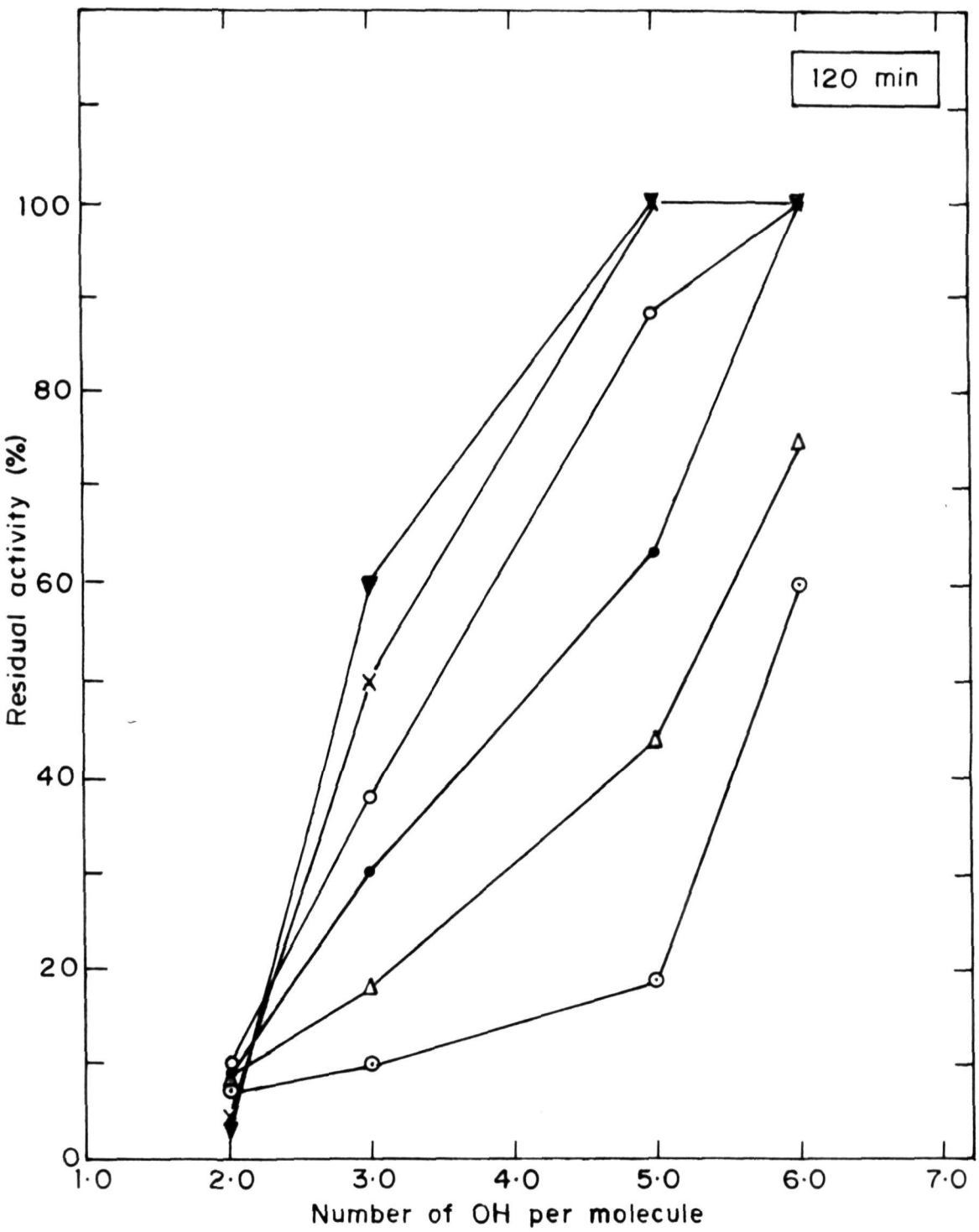

420 min
100
80
60
40
20
0
Residual activity (%)
0
1·0
2·0
3·0
4·0
5·0
6·0
Concentration (M)

Fig. 3.3: Effect of the length of the polyhydric alcohol molecule on the thermostability of pullulan-hydrolysing activity at the end of 120 min. Inactivation was performed at 60°C, 400 $\mu g.ml^{-1}$ enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

Concentration of the polyhydric alcohol:

(o——o) 0.5 M
(Δ——Δ) 1.0 M
(•——•) 1.5 M
(o——o) 2.0 M
(×——×) 3.0 M
(▼——▼) 4.0 M


120 min
Residual activity (%)
0
20
40
60
80
100
1·0
2·0
3·0
4·0
5·0
6·0
7·0
Number of OH per molecule

to the increased thermostability of the pullulan-hydrolysing activity at 60°C (Fig. 3.4).

3.3.2 Discussion

In case of the industrial enzymes, there is often a need to use elevated temperatures to accelerate the rates of enzyme catalyzed reactions. This is especially true for the starch-hydrolytic processes, where temperatures of the order of 60°C are used for extended periods of time. The increase in the reaction rates achieved by increasing the reaction temperature, invariably results in an enhanced rate of the thermal inactivation of the enzyme. As a result, ways and means need to be devised to protect these enzymes.

Among the various methods available for the enhancement of stability of the soluble enzymes, the addition of different external compounds is an important conventional means. Additives that modify the structure of water or strengthen the hydrophobic interactions in the protein molecule (polyhydric alcohols) are often described as stabilizing agents in case of enzymes (Klibanov, 1983; Ye *et al.*, 1988; Graber and Combes, 1989; Asther and Meunier, 1990). The thermostabilizing effect of different polyols has been described for the α -amylase (Graber and Combes, 1989; Asther and Meunier, 1990); rulactine, a proteolytic enzyme (Yoovidhya *et al.*, 1986) and glucose oxidase (Ye *et al.*, 1988).

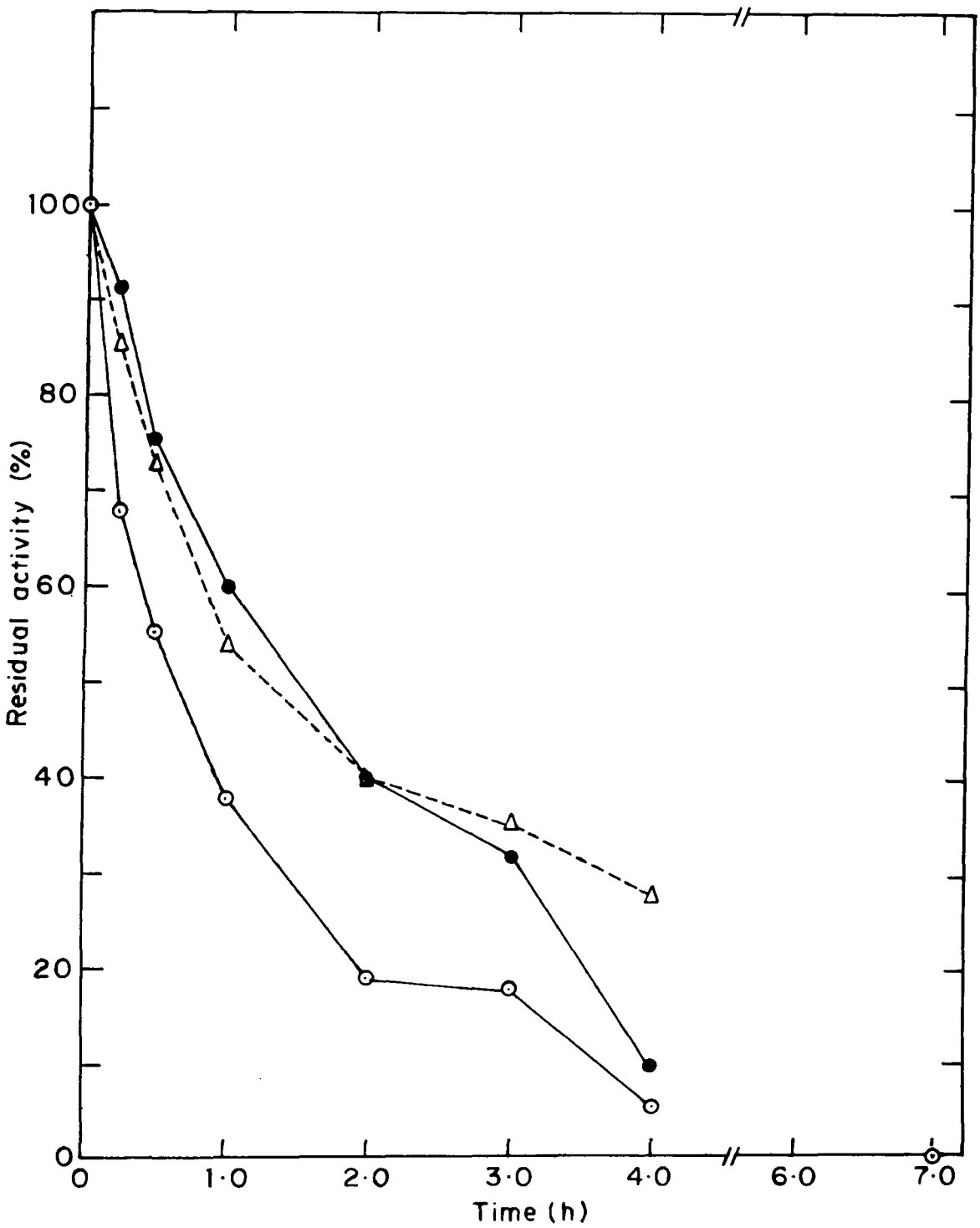
In case of the pullulan-hydrolysing activity from *S. rolfsii*, the stabilizing effect of the polyhydric alcohols was observed to increase with increase in the the chain length of the alcohols tested (Fig. 3.2 a - f and Fig. 3.3). These results are similar to the ones observed in the case of α -amylase from *Aspergillus oryzae* (Graber and Combes, 1989) and *Bacillus licheniformis* (Asther and Meunier, 1990). In case of the glucose oxidase from *A. niger*, similar protective effect was observed with the exception

Fig. 3.4: Effect of addition of substrate on the thermostability of pullulan-hydrolysing activity. Inactivation was carried out at 60°C, 400 μg.ml^{-1} enzyme; residual pullulan-hydrolysing activity was measured as described in section 2.2.10.

(⊙——⊙) Control

(●——●) Pullulan (5 mg.ml^{-1})

(△——△) Pullulan (25 mg.ml^{-1})


Residual activity (%)
100
80
60
40
20
0
0
1·0
2·0
3·0
4·0
6·0
7·0
Time (h)

of sorbitol, which had a less protective effect as compared to erythritol and xylitol (Ye *et al.*, 1988). Dissimilar observations have also been recorded in the case of "rulactine", a protease from *Micrococcus caseolyticus* by Yoovidhya *et al.* (1986). It was observed by them that the protective effect of the polyols decreased with the increasing chain length of the polyol used (ethylene glycol to sorbitol).

Out of the five polyhydric alcohols tested, sorbitol and xylitol had the most beneficial effect towards the stabilization of the enzyme activity. Out of these two alcohols, sorbitol (from a concentration of 1.5 M) was found to provide a complete protection to the enzyme activity. Glycerol and ethylene glycol were found to be very poor substitutes. In fact, rapid inactivation of the enzyme activity in presence of ethylene glycol was observed. Similar effects have also been noted for the α -amylase from *B. licheniformis* (Asther and Meunier, 1990). It has been suggested by Bello and Bello (1976) that the presence of contaminating aldehydes, even in the refined grades of ethylene glycol can lead to such inactivation.

The mechanism of stabilization of different enzymes by the presence of polyhydric alcohols is not yet clear (Klibanov, 1983). It has been reported that the addition of the polyols to aqueous solutions of enzymes strengthens the hydrophobic interactions among the non-polar amino acid residues making them more resistant to unfolding and thermal denaturation (Back *et al.*, 1979). Recently, an alternative explanation has been proposed for the thermostability of the α -amylase from *A. oryzae* (Graber and Combes, 1989). They reported that polyols act as competitive inhibitors of α -amylase and the interaction between the polyols and the active site of the enzyme was a factor that contributed to the stabilization of the enzyme.

In conclusion, it can be said that among the various polyols tested, sorbitol had the most thermostabilizing effect on the pullulan-hydrolysing activity. The use of polyols to alter the microenvironment of the enzyme appeared to be very promising. Despite its empirical character, this classical approach to the thermostabilization is useful from the application view point of the enzyme.

CHAPTER IV

PURIFICATION AND CHARACTERIZATION OF THE PULLULAN-HYDROLYSING ACTIVITY FROM S. rolfsii

SUMMARY

The pullulan-hydrolysing enzyme from the culture filtrates of *S. rolfsii* grown on soluble starch as a carbon source has been purified. The methods used included ultrafiltration (Amicon, PM-10), ion-exchange chromatography (DEAE-Cellulose DE-52) and gel filtration chromatography (Bio-Gel P-150). The enzyme moved as a single band in non-denaturing polyacrylamide gel electrophoreses carried out at pH 2.9 and 7.5. Under denaturing conditions (SDS-polyacrylamide gel electrophoresis) and also in analytical polyacrylamide gel isoelectric focusing (pH 3.5 - 10), the enzyme appeared as a single band. The molecular weight of the enzyme was estimated to be 64,000 daltons by SDS-PAGE and 66,070 daltons by gel filtration on Bio-Gel P-150. The enzyme hydrolysed pullulan optimally at 50°C between pH 4.0 - 4.5, whereas, soluble starch was optimally hydrolysed at a pH of between 4.0 - 4.5 and at 65°C. The Michaelis constant (K_m) for pullulan was 5.13 $mg.ml^{-1}$ (V_{max} 1.0 $U.mg^{-1}$) and for soluble starch, it was 0.6 $mg.ml^{-1}$ (V_{max} 8.33 $U.mg^{-1}$). The enzyme was observed to be a glycoprotein (12 - 13% carbohydrate by weight) and had a strong affinity for Concanavalin A. Acarbose, an oligosaccharide analog, was found to be a potent inhibitor of both pullulan and starch hydrolysis. It completely inhibited the action of the enzyme at very low concentrations (0.06 μM) on pullulan and starch. The enzyme showed hydrolytic action on a number of α -D-glucans in an exo-hydrolytic mode, which resulted in the release of glucose as the sole product of hydrolysis. Based on the data about its substrate specificity and mode of action, the enzyme activity was characterized as a glucoamylase (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3).

CHAPTER IV

4.1 INTRODUCTION

Microbial exopolysaccharides have an inherent advantage of their amenability to biodegradation which facilitates their easy recycling. Pullulan, an exopolysaccharide produced by *Aureobasidium pullulans* is thus susceptible to microbial enzymatic attack. Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) specifically hydrolyses the α-(1->6)-glycosidic linkages in pullulan, producing maltotriose. Since the first report of Bender and Wallenfels (1961), who discovered the enzyme in *Aerobacter aerogenes* (*Klebsiella oxytoca*, *K. pneumoniae*), several microbial pullulanases have been purified and characterized (Walker, 1968; Mercier *et al.*, 1972; Nakamura *et al.*, 1975; Plant *et al.*, 1986; Odibo and Obi, 1988). The present understanding of the pullulan-hydrolysing enzymes of microbial origin has made the area quite complex. This has been because of the identification of at least four more classes of pullulan-hydrolysing enzymes (Sakano *et al.*, 1972; Kuriki *et al.*, 1988a; Saha and Zeikus, 1989; Fagerström *et al.*, 1990).

There are few reports of fungal enzymes capable of hydrolysis of pullulan in literature (Sakano *et al.* 1972; McCleary and Anderson, 1980; DeMot and Verachtert, 1987). Hence, the identification of *S. rolfsii* as a potential source of pullulan-hydrolysing activity was significant (Kelkar *et al.*, 1988). So, purification and characterization of the enzyme activity was undertaken.

4.2 RESULTS

4.2.1 Preparation of culture filtrate

S. rolfsii was grown on an optimized NM-2 medium containing soluble starch (1% w/v) as the carbon source as described in section 2.2.2. After cultivation for 10 days at 30°C, the mycelium was harvested from the fermentation broth by decantation and filtration of the supernatant through a pad of glass wool. After harvesting the culture filtrate the pH was adjusted to between 4.5 - 5.0 (initial pH at the time of harvest is 3.0) by tri-sodium citrate (1 M). The culture supernatant was stored at -15°C after the addition of sodium azide (0.01% w/v) as a preservative.

4.2.2 Enzyme purification

The following operations were carried out at 0 - 8°C, unless otherwise indicated. Analytical polyacrylamide gel electrophoresis was used to follow the enzyme purity. Pullulan-hydrolysing activity , starch-hydrolysing activity and protein assays were used for monitoring the column effluents as described in section 2.3.6.

4.2.2.1 Ultrafiltration

Since the culture filtrate obtained after harvesting had a low concentration of protein (~ 400 $\mu g.ml^{-1}$), it was subjected to concentration by ultrafiltration (Amicon, PM-10 membrane, 350 ml capacity). Ten times concentrated (of the initial volume) culture filtrate gave recovery of the enzyme activities around 65%.

4.2.2.2 DEAE-Cellulose DE-52 chromatography

Enzyme concentrated by ultrafiltration was dialysed extensively against 0.05 M acetate buffer (pH 4.9) (column buffer). Before loading on to the column, it was clarified by centrifugation (8000 xg, 20 min). The dialysed enzyme was then

chromatographed on a column of DEAE-Cellulose DE-52 (2 X 10 cm). The column was developed at a rate of 12 - 13 $ml.h^{-1}$. After the enzyme loading was complete, the column was washed with the column buffer with at least four times the bed volume. Elution of the enzyme activity was then carried out with a linear salt gradient (0 - 0.4 M NaCl, 300 ml) in the column buffer. Fractions (4 ml) were processed for the determination of the enzyme activities and protein content. The elution pattern is shown in Fig. 4.1. It was observed that the pullulan, as well as the starch hydrolysing activities eluted in the same fractions. Fractions (25 to 29) were pooled and dialyzed against the column buffer and concentrated by lyophilization.

4.2.2.3 Bio-Gel P-150 chromatography

The concentrated enzyme from the previous step was dissolved in 1.5 - 2.0 ml of the column buffer and subsequently loaded on a column of Bio-Gel P-150 (1 X 100 cm). Fractions (2.5 ml) were collected at a flow rate of 8 $ml.h^{-1}$. The elution profile observed is given in Fig. 4.2. The recovery of the enzyme activity was 22.1%.

The yield and specific activity of enzymes at different stages of purification are summarized in Table 4.1.

4.2.3 Criteria of purity

The pullulan-hydrolysing enzyme in both cathodic (2.9) and anodic (7.5) polyacrylamide disc gel electrophoreses showed a single band (Fig. 4.3 a,b). Electrophoresis of the enzyme under denaturing conditions (SDS-PAGE) also gave a single band (Fig.4.4 a). The analytical isoelectric focusing in polyacrylamide gel (7.5%, pH 3.5 - 10.0) of the enzyme gave a single band (Fig. 4.4 b).

Fig. 4.1: Ion-exchange chromatography on DEAE-Cellulose DE-52. Amicon concentrated enzyme (PM-10 membrane; 2.5 $mg.ml^{-1}$; 25 ml) adjusted to pH 4.9 by extensive dialysis against 0.05 M acetate buffer (column buffer) was applied to the column (2 X 10 cm), previously equilibriated with the column buffer. Elution of the bound enzyme was achieved with a linear gradient of 0.0 - 0.4 M NaCl in column buffer (300 ml) at a rate of 12 $ml.h^{-1}$. Fraction volume was 4 ml.

(⊙——⊙) Pullulan-hydrolysing activity ($IU.ml^{-1}$)

(o——o) Starch-hydrolysing activity ($IU.ml^{-1}$)

(•——•) Protein ($mg.ml^{-1}$)

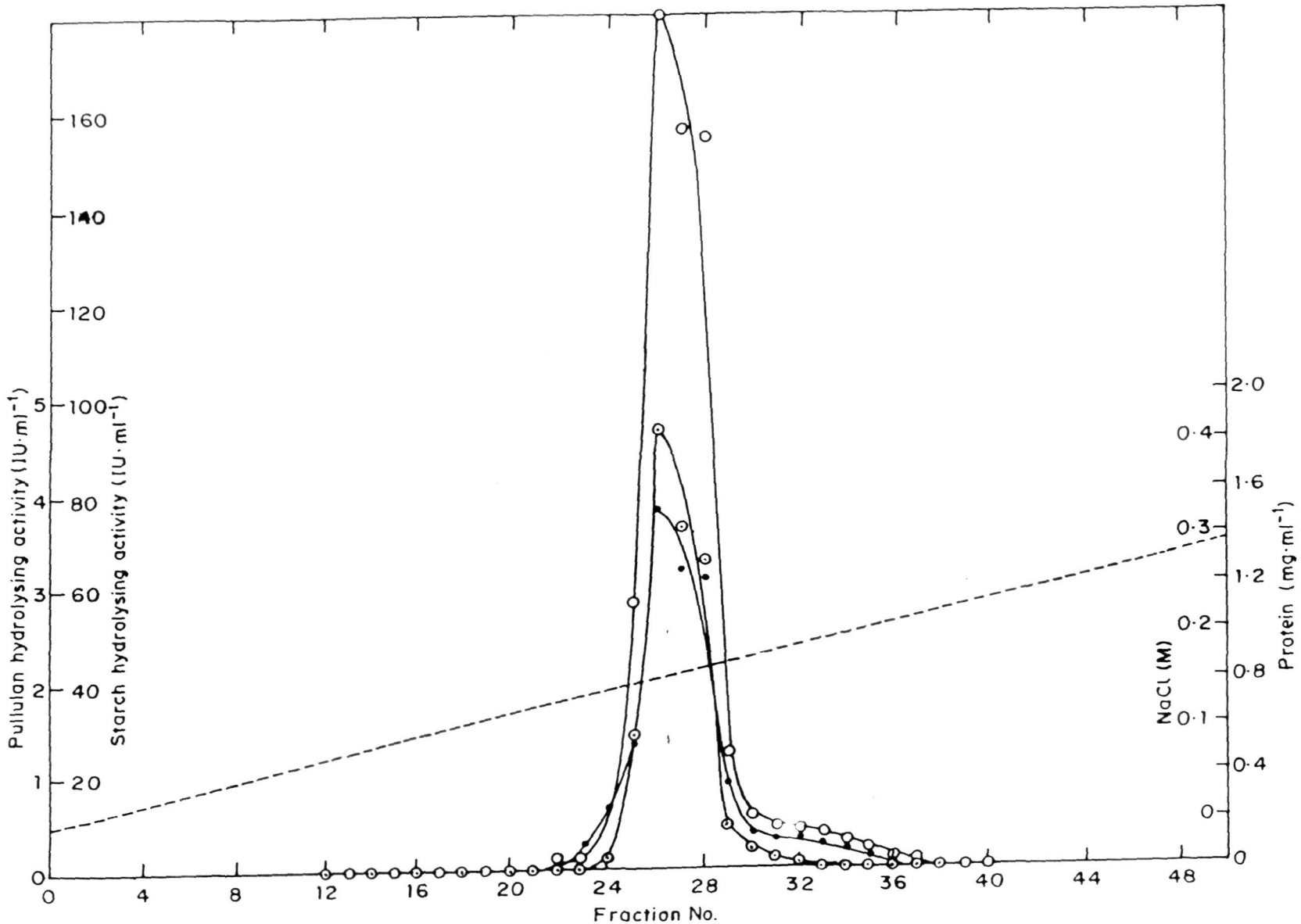

Pullulan hydrolysing activity (IU·ml⁻¹)
Starch hydrolysing activity (IU·ml⁻¹)
NaCl (M)
Protein (mg·ml⁻¹)
Fraction No.
0
4
8
12
16
20
24
28
32
36
40
44
48
20
40
60
80
100
120
140
160
1
2
3
4
5
0·1
0·2
0·3
0·4
0·8
1·2
1·6
2·0

Fig. 4.2: Gel filtration chromatography on Bio-Gel P-150. The pooled fractions from the DEAE-cellulose chromatography (lyophilized to 1 ml, 15 $mg.ml^{-1}$) was applied to a column of Bio-Gel P-150 (1 X 100 cm) previously equilibriated with 0.05 M acetate buffer (pH 4.9) (column buffer). Fractions of 1.6 ml were collected at a flow rate of 8 $ml.h^{-1}$.

(⊙——⊙) Pullulan-hydrolysing activity ($IU.ml^{-1}$)

(○——○) Starch-hydrolysing activity ($IU.ml^{-1}$)

(•——•) Protein ($mg.ml^{-1}$)

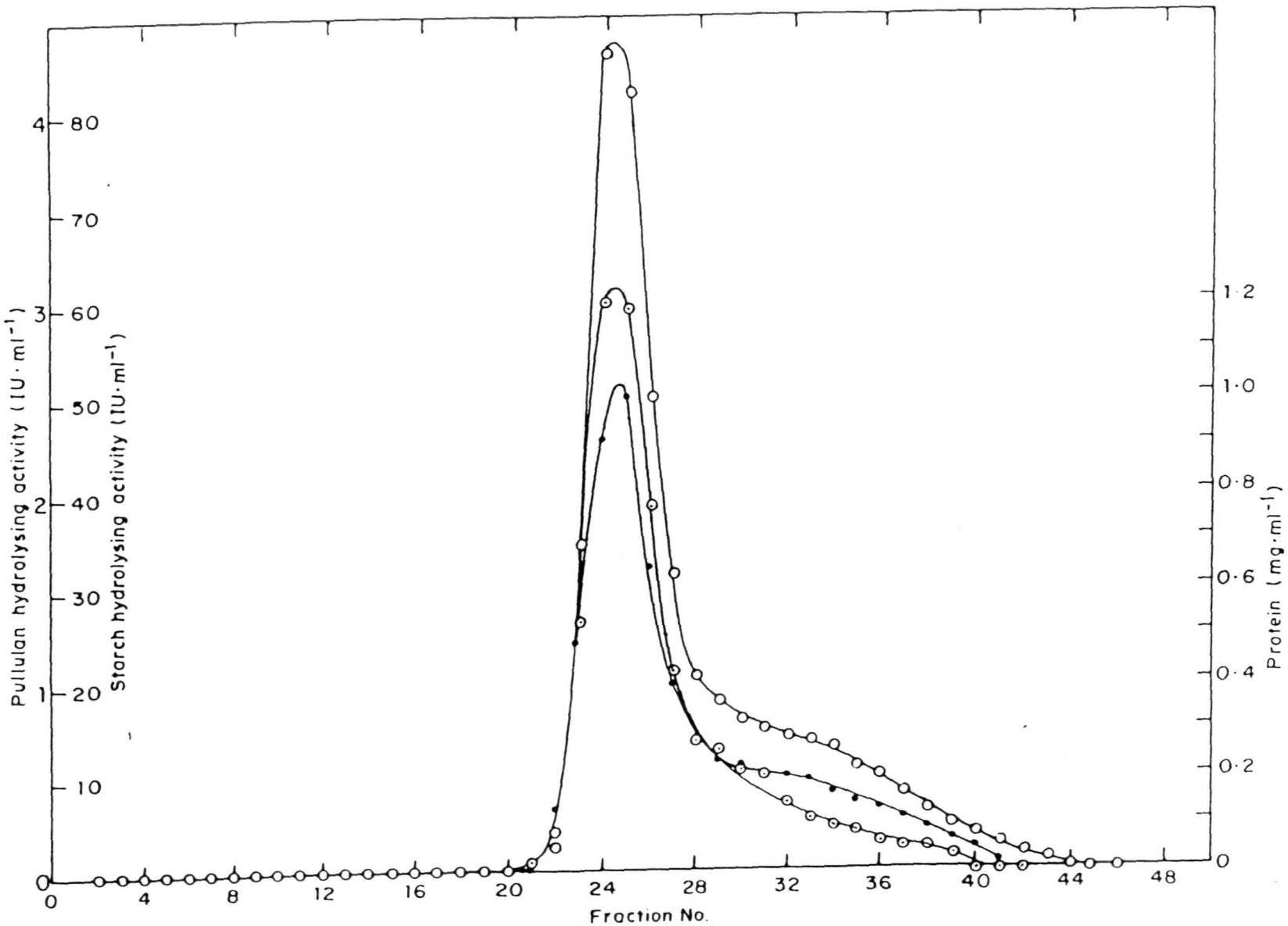

Pullulan hydrolysing activity (IU·ml⁻¹)
Starch hydrolysing activity (IU·ml⁻¹)
Protein (mg·ml⁻¹)
Fraction No.
0
4
8
12
16
20
24
28
32
36
40
44
48
10
20
30
40
50
60
70
80
1
2
3
4
0·2
0·4
0·6
0·8
1·0
1·2

Table 4.1: Summary of purification of pullulan-hydrolysing enzyme from *S. rolfsii* culture filtrate

Step	Total Protein (mg)	Pullulan-hydrolysing activity			Starch-hydrolysing activity			Glucoamylase activity		
		Total Units	Specific activity	% Recovery	Total units	Specific activity	% Recovery	Total units	Specific activity	% Recovery
1. Culture filtrate	236.5	184.5	0.78	100	6025	25.47	100	5265	22.20	100
2. Amicon (PM-10)	128.0	125.0	0.98	67.7	4010	31.33	66.6	3360	26.25	63.8
3. DEAE-Cellulose DE-52 (0-0.4 M NaCl)	30.0	71.2	2.37	38.6	2254	75.13	37.4	2030	67.66	38.6
4. Bio-Gel P-150	13.6	40.8	3.00	22.1	1447	106.5	24.0	1219	89.75	23.2

Fig. 4.3: (a) Analytical polyacrylamide gel electrophoresis of the pullulan-hydrolysing enzyme at pH 7.5. Arrow marks the position of the marker dye.

(b) Analytical polyacrylamide gel electrophoresis of the pullulan-hydrolysing enzyme at pH 2.9. Arrow marks the position of the marker dye.

Fig. 4.3 a

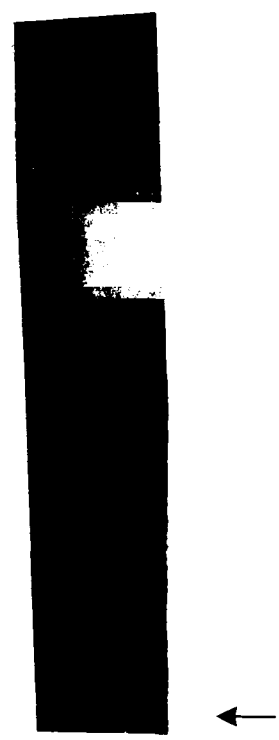


Fig. 4.3 b

Fig. 4.4: (a) Migration of the enzyme in SDS-polyacrylamide gel electrophoresis (7.5%) after treatment (100°C, 5 min) with 1% (w/v) SDS in combination with 1% (v/v) 2-mercaptoethanol according to Weber and Osborn (1969). Lane 1 contains standard protein markers. Lanes 2 and 3 contain enzyme from *S. rolfsii*.

(b) Isoelectric focusing in polyacrylamide gel (7.5%) over a pH gradient of 3.5 - 10.0.

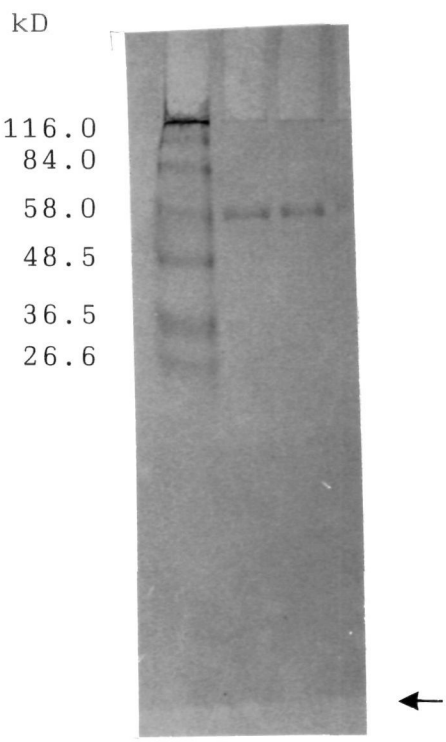


Fig. 4.4 a

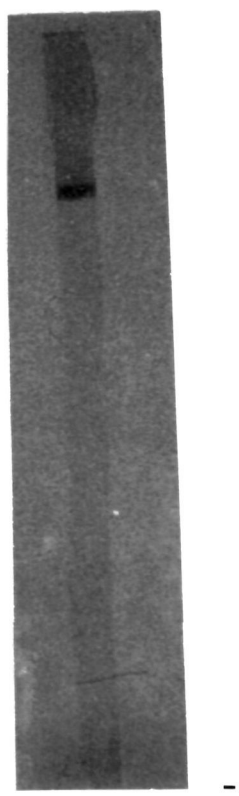


Fig. 4.4 b

4.2.4 Determination of the molecular weight

4.2.4.1 Gel filtration

Molecular weight of the purified enzyme was estimated by the molecular sieve chromatography method of Andrews (1964). A column of Bio-Gel P-150 (1 X 100 cm) was equilibriated in 0.05 M acetate buffer (pH 4.9).

A plot of V_e/V_o against the logarithm molecular weights was plotted according to Andrews (1964) (Fig. 4.5). The molecular weight of the pullulan-hydrolysing activity was found to be 66,070.

4.2.4.2 SDS-polyacrylamide gel electrophoresis (molecular weight and the subunit structure)

The estimate of the molecular weight of the enzyme was made by its migration in the SDS-PAGE according to the method of Weber and Osborn (1969). The enzyme appeared as a single band after treatment with 1% SDS in combination with 1% 2-mercaptoethanol and/or 1% iodo-acetamide indicating that it was probably composed of a single polypeptide chain. A plot of the logarithm of the molecular weight markers used (SDS-prestained marker kit, Sigma Chemical Co.) versus their relative mobilities was plotted. An estimate of the molecular weight of the enzyme was 64,000 daltons as obtained from the calibration curve (Fig. 4.6).

4.2.5 Glycoprotein nature

The enzyme appeared to have 12-13 % saccharides associated with it when estimated by the phenol-H_2SO_4 method (Section 2.3.2), indicating that the enzyme was a glycoprotein. The glycoprotein nature of the enzyme was then further confirmed by the following criteria:

Fig. 4.5: Molecular weight determination of the enzyme by gel filtration. A Bio-Gel P-150 column (1 X 100 cm) was equilibriated with 0.05 M acetate buffer (pH 4.9). The calibration of the column was done with (1) alcohol dehydrogenase (141,000); (2) bovine serum albumin (67,000); (3) ovalbumin (43,000); (4) soybean trypsin inhibitor (21,000); (5) lysozyme (14,300).

V_O, void volume; V_e, elution volume

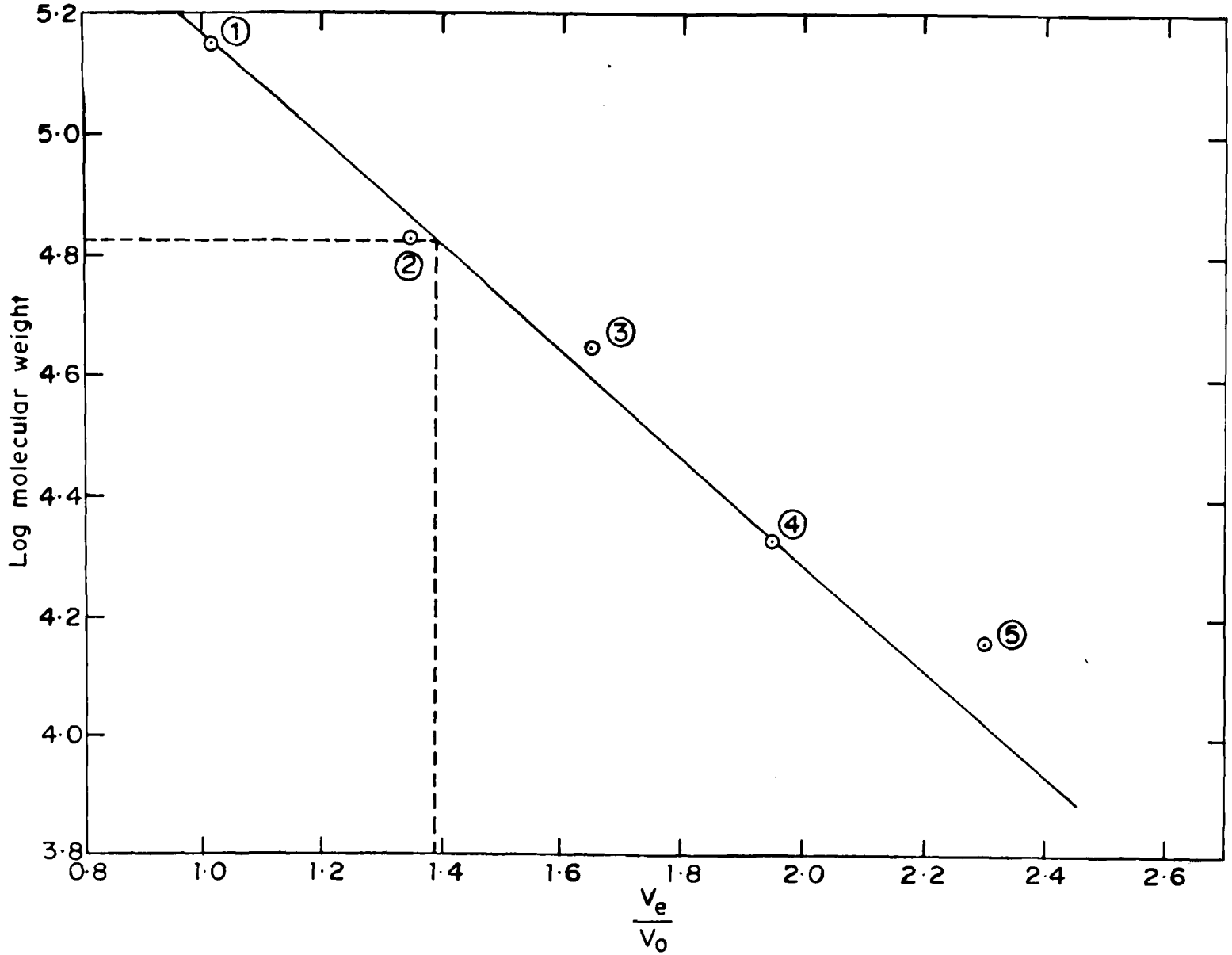
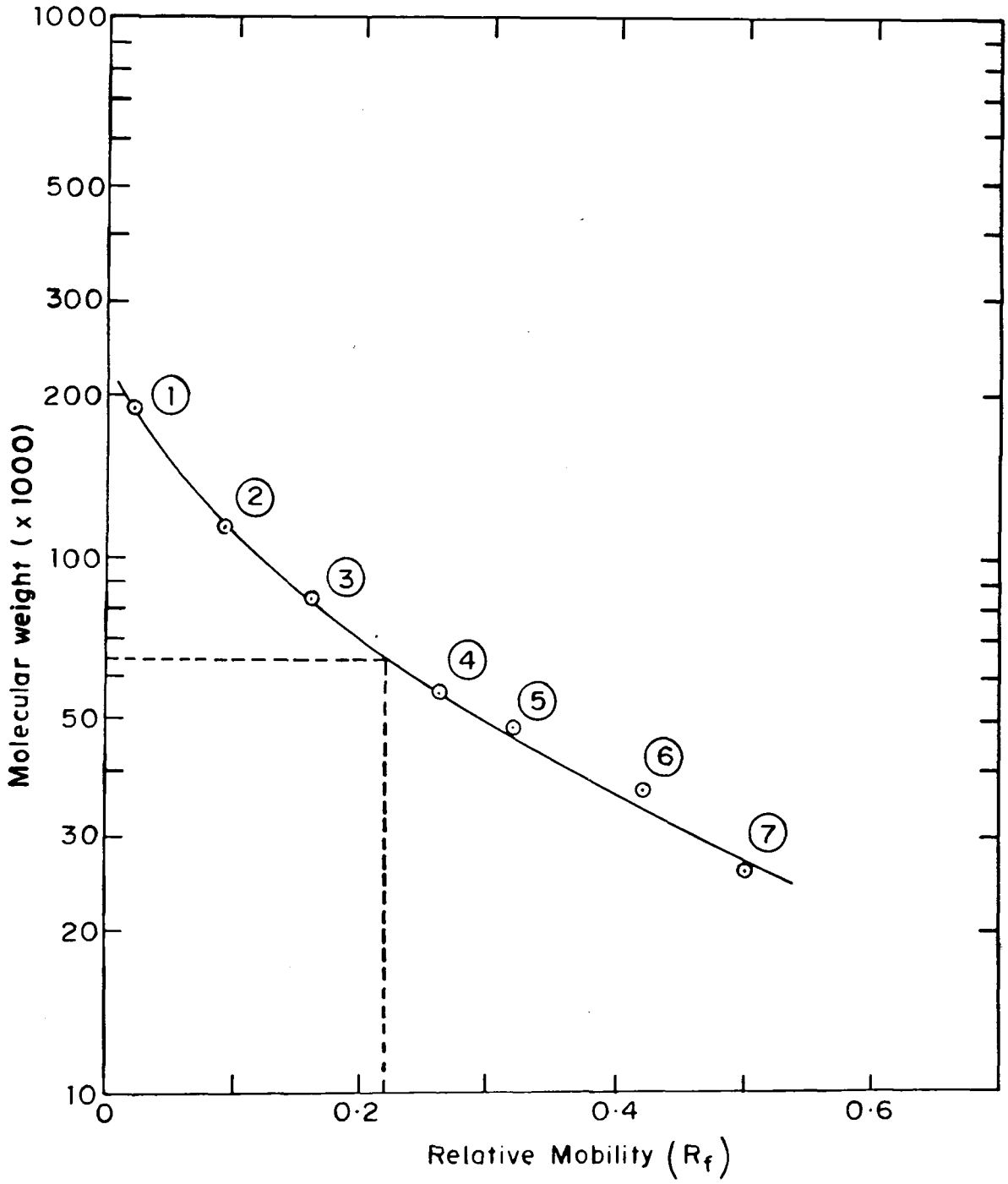

5·2
5·0
4·8
4·6
4·4
4·2
4·0
3·8
Log molecular weight
0·8
1·0
1·2
1·4
1·6
1·8
2·0
2·2
2·4
2·6
Ve/Vo
①
②
③
④
⑤

Fig. 4.6: Molecular weight determination of the enzyme by SDS-polyacrylamide gel electrophoresis (7.5%). Relative mobilities of the standard marker proteins were plotted against the molecular weights. The marker proteins used were (1) α_2-macroglobulin (180,000); (2) β-galactosidase (116,000); (3) fructose 6-phosphate kinase (84,000); (4) pyruvate kinase (58,000); (5) fumarase (48,500); (6) lactic dehydrogenase (36,500) and (7) triosephosphate isomerase (26,600).


1000
500
300
200
100
50
30
20
10
Molecular weight (x 1000)
0
0·2
0·4
0·6
Relative Mobility (R_f)
1
2
3
4
5
6
7

The enzyme had a very strong affinity for concanavalin A. When chromatographed on a column of Concanavalin A-Sepharose CL-4B (0.8 X 8 cm, 0.05 M acetate buffer, pH 4.9 containing 0.002 M each of $MgCl_2$, $MnCl_2$ and $CaCl_2$), the enzyme was found to be adsorbed on the column as no activity could be detected in the effluent. The specific elution of the enzyme activity was achieved by using a linear gradient of α-methyl-D-mannopyranoside (0-1 M) in the same buffer. The enzyme activity eluted as a single peak between 0.6-0.8 M concentration of α-methyl-D-mannopyranoside.

Polyacrylamide gels stained for glycoproteins by the thymol-H_2SO_4 method (Gander, 1984) showed a single dark-brown band, while an identical gel stained for protein gave a corresponding band.

4.2.6 Properties of the enzyme

4.2.6.1 Stability of the purified enzyme

The purified enzyme was stable when stored at -15°C at pH 4.9, with no significant loss of activity over a 12-months period.

4.2.6.2 Isoelectric pH

The isoelectric pH of the enzyme was determined to be 4.3 using preparative isoelectric focusing in sucrose/glycerol gradients and isoelectric focusing in polyacrylamide gels.

4.2.6.3 Optimum pH for activity

The pH dependence of pullulan and starch hydrolysis was examined by measuring the reducing sugar production at different pH values (Fig. 4.7, 4.8). The pH range was covered by 0.1 M glycine - HCl buffer (pH 2.0 - 3.5); 0.1 M sodium

Fig. 4.7: Effect of pH on the rate of hydrolysis of pullulan at 50°C by the *S. rolfsii* enzyme.

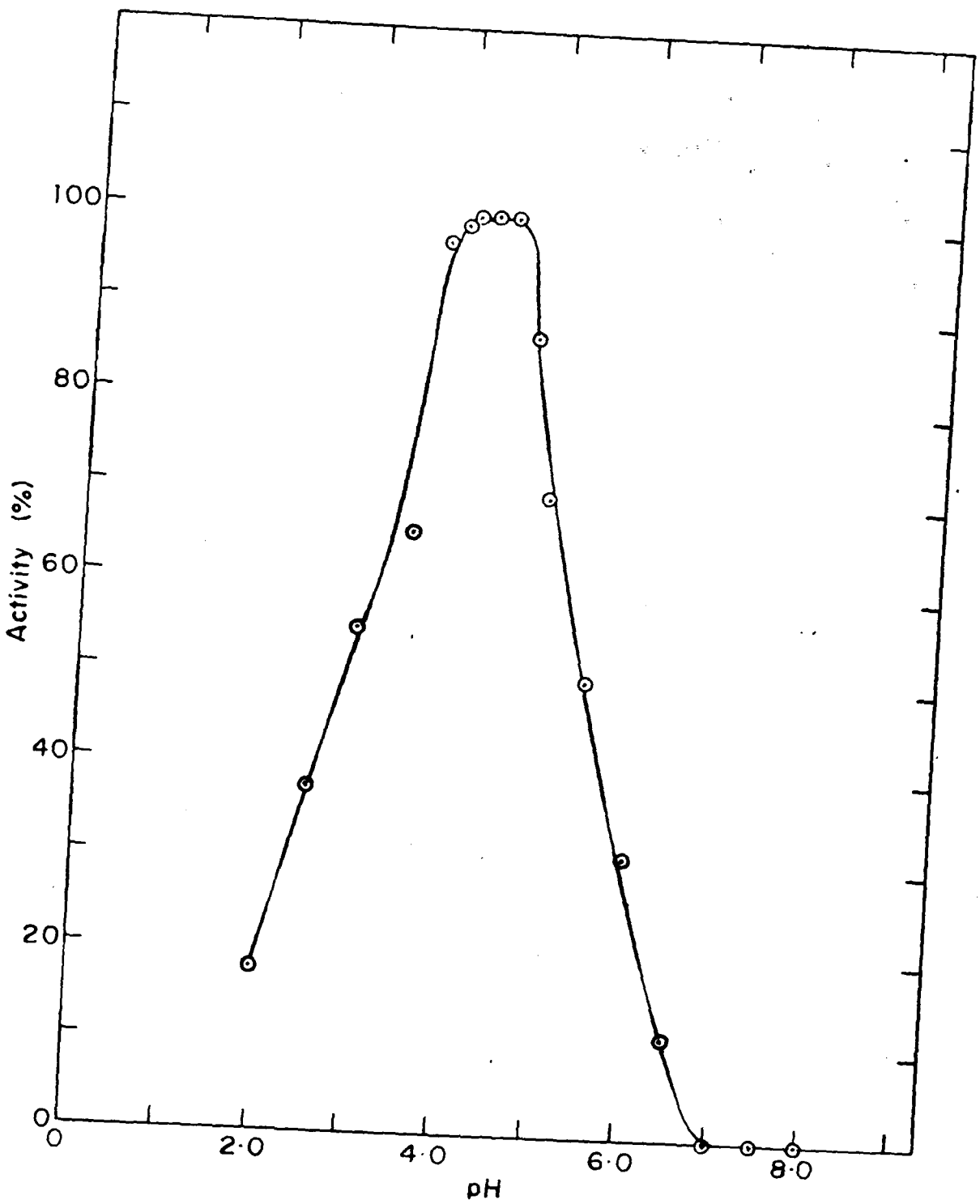
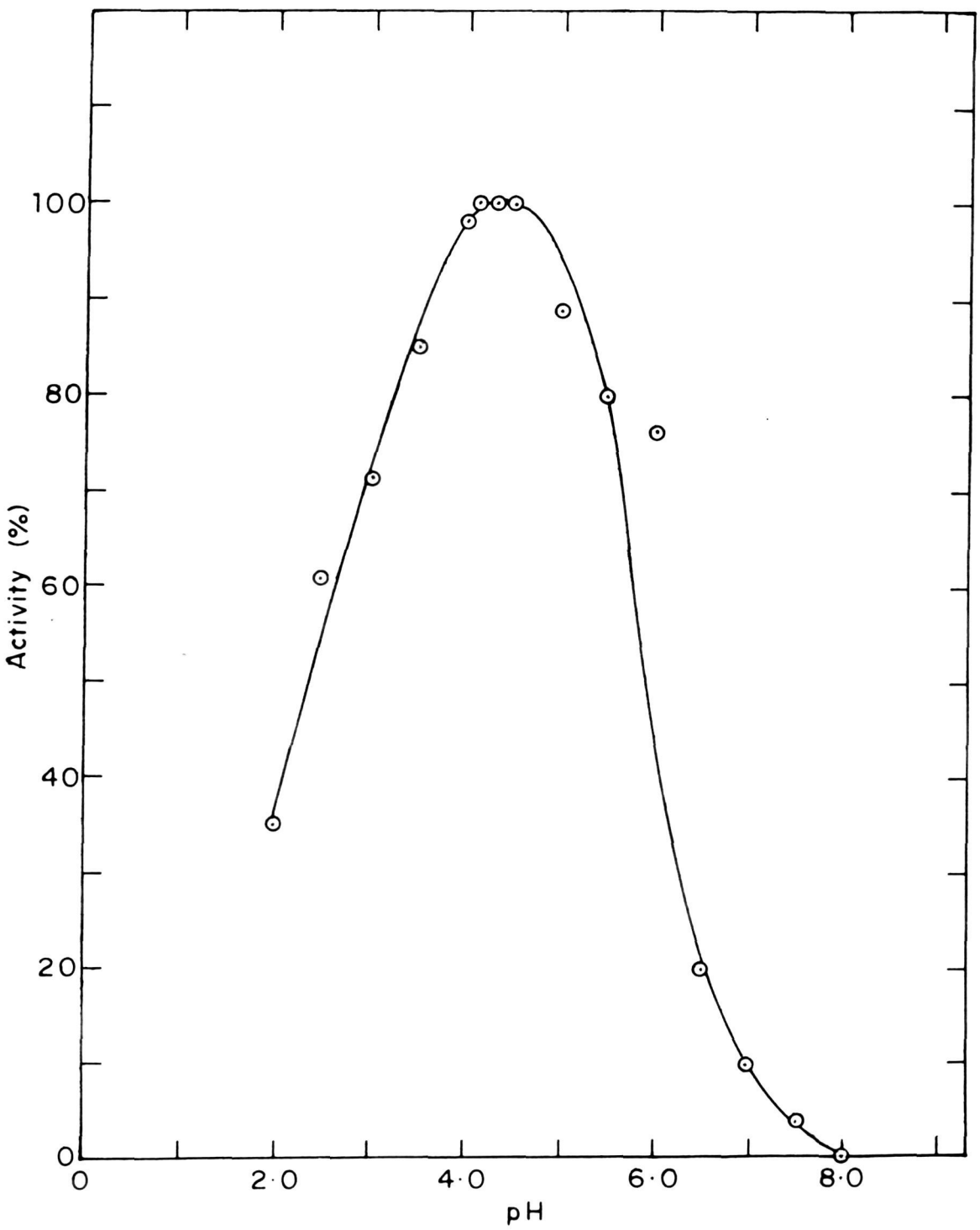

Activity (%)
100
80
60
40
20
0
0
2·0
4·0
6·0
8·0
pH

Fig. 4.8: Effect of pH on the rate of hydrolysis of soluble starch at 50°C by the *S. rolfsii* enzyme.


100
80
60
40
20
0
Activity (%)
0
2·0
4·0
6·0
8·0
pH

acetate - acetic acid buffer (pH 4.0 - 6.0) and 0.1 M sodium phosphate buffer (pH 6.5 - 8.0). The optimum pH for the hydrolysis of both pullulan and starch was observed to be between 4.0 - 4.5.

4.2.6.4 Optimum temperature for activity

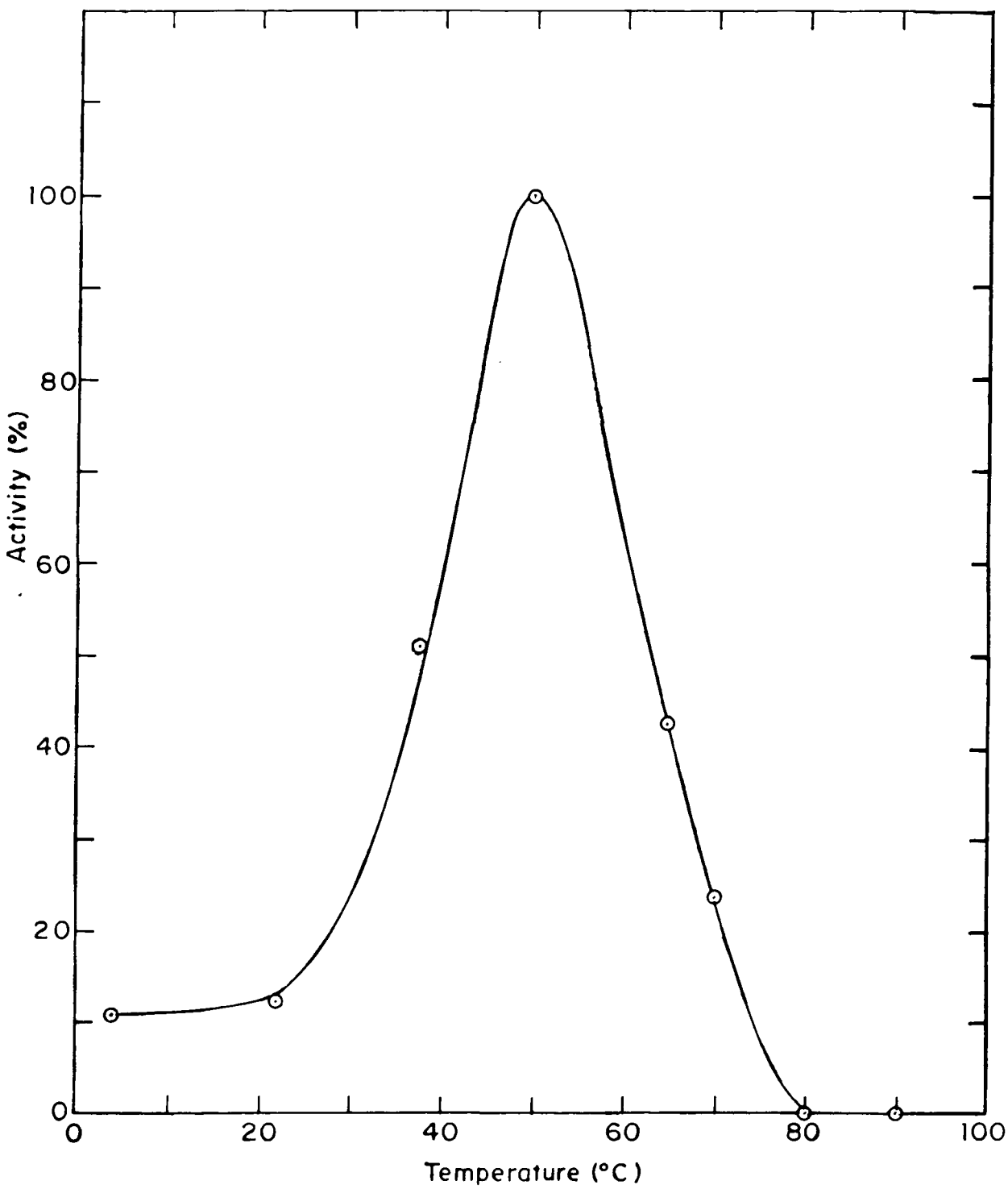
The effect of temperature on the pullulan and starch hydrolysis was determined using the standard assay system. The temperature of the assay was varied between 22 and 90°C, using 0.05 M acetate buffer (pH 4.9) (Fig. 4.9, 4.10). The optimum temperature for the hydrolysis of pullulan was found to be 50°C, whereas for the hydrolysis of starch it was 65°C. The activation energy for the hydrolysis of pullulan was determined to be 19.8 $kJ.mol^{-1}$ from the Arrhenius plots, whereas for the hydrolysis of starch the activation energy was estimated at 25.9 $kJ.mol^{-1}$ (Fig. 4.11, 4.12).

4.2.6.5 Stability of the enzyme at different pH

For the determination of the stability of the pullulan and starch hydrolysing activities, the enzyme samples were adjusted to different pH in the range of 2.5 - 8.0 by the addition of 0.05 M of suitable buffer. The pH range was covered by the Glycine - HCl buffer (pH 2.6 - 3.3), sodium acetate - acetic acid (pH 4.1 - 4.8) and sodium phosphate buffer (pH 6.0 - 8.0). After incubating the enzyme for 15 min at 50°C, the residual starch- and pullulan-hydrolysing activity was determined under standard assay conditions. The residual activities were compared to that of a control maintained at 4°C at pH 4.5 for the same duration.

Fig. 4.9: Effect of temperature on the rate of hydrolysis of pullulan by the *S. rolfsii* enzyme (0.05 M acetate buffer, pH 4.9).

Time ?


Activity (%)
0
20
40
60
80
100
0
20
40
60
80
100
Temperature (°C)

It was observed that the pullulan-hydrolysing activity of the enzyme was more stable in the pH range of 3.5 - 5.0 (Fig. 4.13, 4.14). Addition of BSA (500 $\mu g.ml^{-1}$) did not not show any benificial effect on the pullulan-hydrolysing activity, however, the starch-hydrolysing activity showed a stabilizing effect in presence of BSA.

4.2.6.6 Thermostability of the enzyme

The samples of the enzyme were incubated at various temperatures (4 - 80°C) in 0.05 M acetate buffer (pH 4.9) for 15 min and then immediately chilled on ice. The residual pullulan- and starch-hydrolysing activities of the enzyme were estimated (Fig. 4.15). The enzyme retained both of the activities to the extent of 90% after 15 min at 50°C. After 80°C, both of the activities were inactivated after 15 min.

4.2.6.7 Km and Vmax

The formation of reducing sugars at different substrate concentrations was estimated in the reaction mixture by the Somogyi-Nelson method. The values of K_m and V_{max}, determined for the respective substrates, are summarized in Table 4.2.

4.2.6.8 Effect of various metal ions/additives on the enzyme activity

For determining the effect of different additives and metal ions, the purified enzyme (1 - 2 μg) was pre-incubated with the additive at 25°C for 15 min (Table 4.3). The effect of various metal ions was determined by the detection of the reducing sugars formed during the assay by the Somogyi-Nelson or the Glucose oxidase-peroxidase methods. The pullulan- and starch-hydrolysing activities of the enzyme were found to be unaffected to a large extent by most of the metal ions (Table 4.3). The metal ions such as Mn^{++}, Mg^{++}, Co^{++}, Ca^{++}, Mo^{++}, Li^{+}, Zn^{++}

Fig. 4.13: Influence of pH on the stability of the pullulan-hydrolysing activity of the *S. rolfsii* enzyme. The enzyme (2 $\mu g.ml^{-1}$) was maintained at 50°C for 15 min at different pH values with BSA or without BSA. Residual pullulan-hydrolysing activity was determined under standard assay conditions.

(⊙——⊙) With BSA (500 $\mu g.ml^{-1}$)

(⊡——⊡) Without BSA

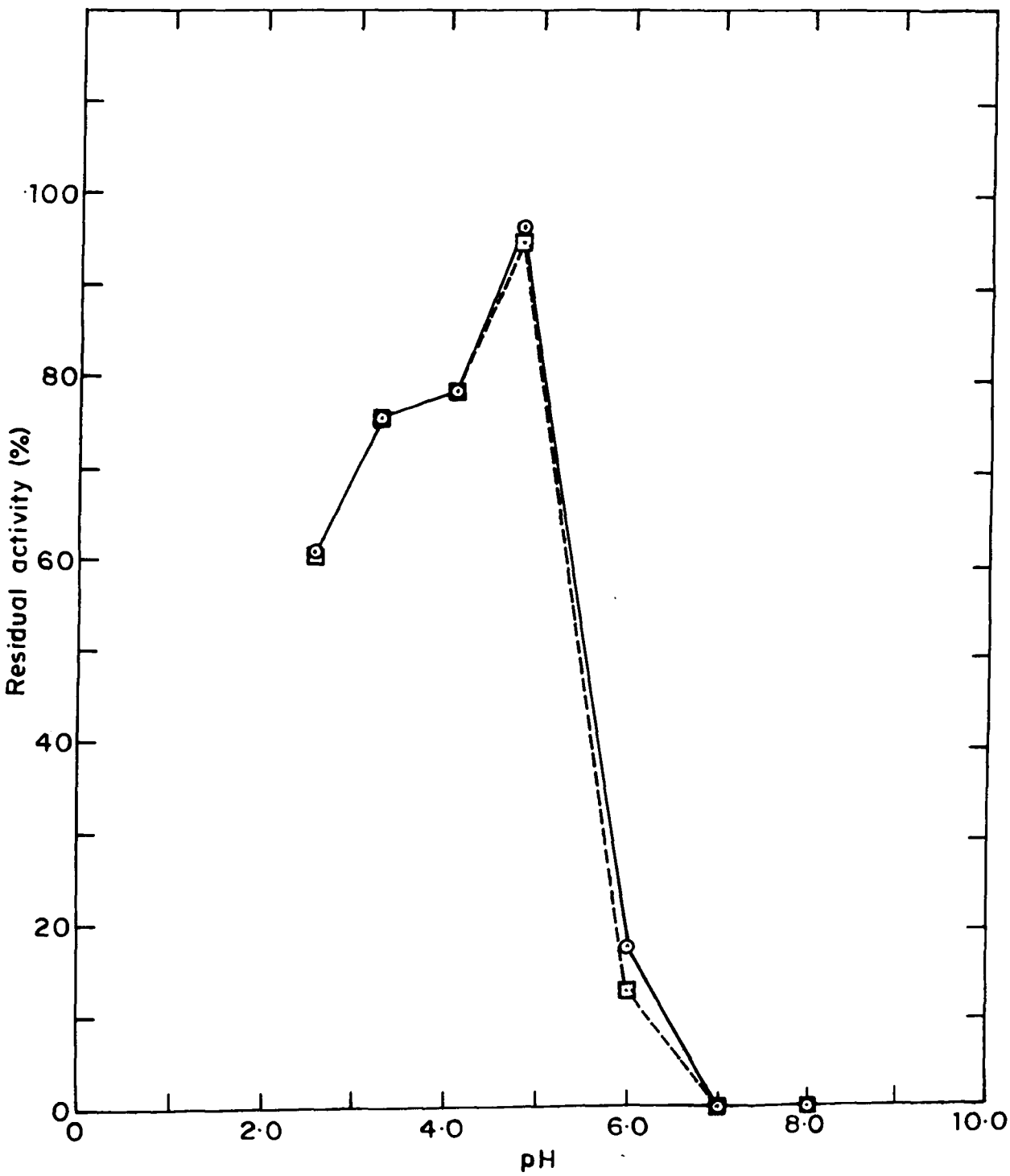

100
80
60
40
20
0
Residual activity (%)
0
2·0
4·0
6·0
8·0
10·0
pH

Fig. 4.14: Influence of pH on the stability of the soluble starch- hydrolysing activity of the *S. rolfsii* enzyme. The enzyme (1 μg.ml^{-1}) was maintained at 50°C for 15 min at different pH values with BSA or without BSA. Residual soluble starch-hydrolysing activity was determined under standard assay conditions.

(⊙——⊙) With BSA (500 μg.ml^{-1})

(⊡——⊡) Without BSA

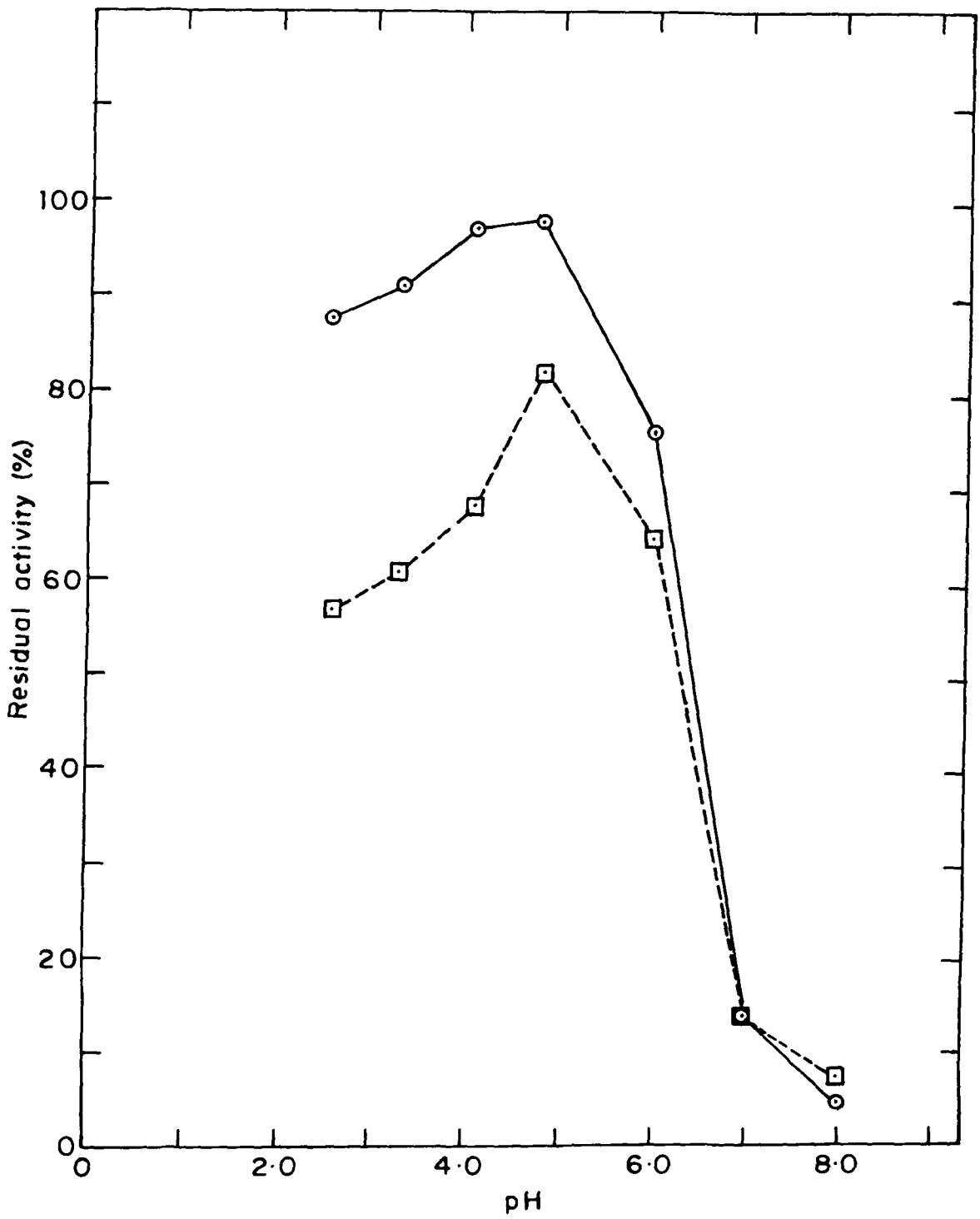

Residual activity (%)
100
80
60
40
20
0
0
2·0
4·0
6·0
8·0
pH

Fig. 4.15: Thermostability of the pullulan- and soluble starch-hydrolysing activity of the *S. rolfsii* enzyme. The enzyme was incubated at various temperatures in 0.05 M acetate buffer (pH 4.9) for 15 min and residual pullulan- and soluble starch-hydrolysing activities determined.

(⊙——⊙) pullulan-hydrolysing activity

(⊡——⊡) soluble starch-hydrolysing activity

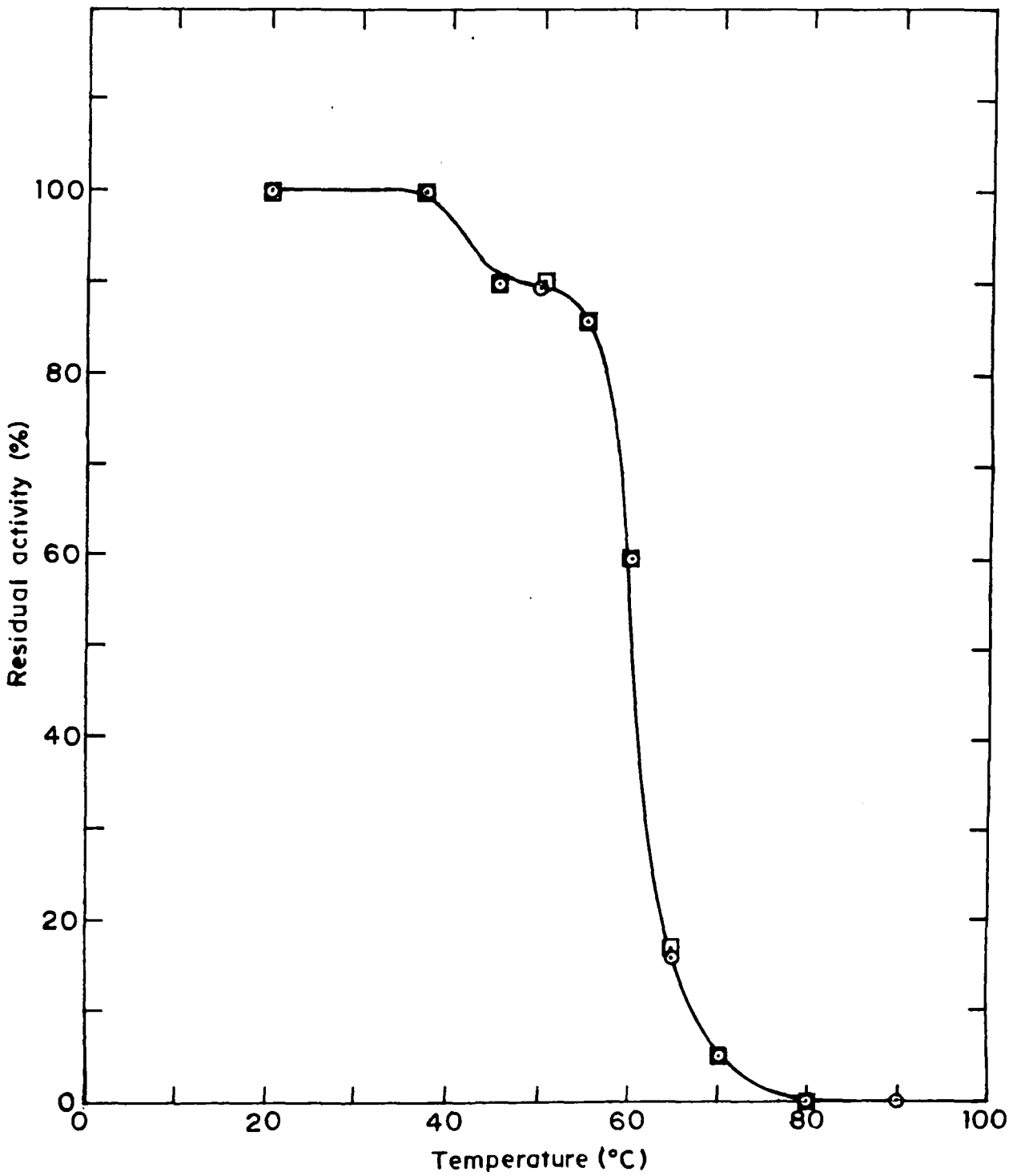

Residual activity (%)
100
80
60
40
20
0
0
20
40
60
80
100
Temperature (°C)

Table 4.2 : K_m and V_{max} for the hydrolysis of different substrates.

Substrate	K_m (mg.ml^{-1})	V_{max} (μ mol of glucose equivalents.min^{-1})
Pullulan	5.13	1.00
Soluble starch	0.60	8.33
Amylose	1.36	9.52
Amylopectin	0.63	9.09
Glycogen	0.42	6.25

Table 4.3 : Effect of various metal ions/additives on the enzyme activity.

Metal Ion/Additive (0.005 M)	Residual Activities (%)	
	Pullulan-hydrolysis	Starch-hydrolysis
Control	100.0	100.0
Magnesium	88.6	88.0
Cobalt	91.0	96.0
Calcium	95.0	88.0
Molybdenum	87.0	85.0
Lithium	92.0	92.0
Arsenic	96.0	89.0
Zinc	93.0	79.0
Copper	44.0	79.0
Mercury	66.0	54.0
Lead	30.0	5.0
Iron	18.0	40.0
Manganese	100.0	100.0
EDTA	70.0	66.0
p-HMB (0.001 M) [a]	N.D.	N.D.

Note : Enzyme preincubated with additive in 0.05 M acetate buffer (pH 4.9) at 25°C for 15 min before determination of the residual activity

N.D. not detected

[a] p-hydroxymercury benzoic acid

and As^{++} inhibited both the enzyme activities to an extent of 10 - 20% only. Metal ions like Cu^{++}, Pb^{++} and Fe^{+++} significantly inhibited both the activities of the enzyme (60 - 80% inhibition).

4.2.6.9 Rate of pullulan and starch hydrolysis

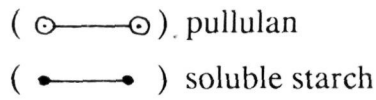
The enzymatic activity on pullulan (2 μg enzyme, 10 mg pullulan) and starch (1 μg enzyme, 10 mg soluble starch) was studied over a period of one hour (Fig. 4.16). It was noted that in case of both of the substrates, reducing groups were liberated exponentially with time.

4.2.6.10 Activity towards different substrates

a. *Initial rate of hydrolysis*

Various α-D-glucans and β-D-glucans were tested for the relative initial rates of hydrolysis by the enzyme. All these assays were performed at 50°C in 0.05M acetate buffer (pH 4.9). The oligosaccharide substrates were used at a concentration of 5 $mg.ml^{-1}$ and the polysaccharide substrates were used at 10 $mg.ml^{-1}$. The results of these experiments are summarized in Table 4.4. It can be seen that the enzyme was most active on polysaccharide substrates like starch, glycogen and dextrin (a hydrolysed starch preparation). It had almost similar activities on amylose and amylopectin. In the series on malto-oligosaccharides, the initial rate of hydrolysis was observed to increase from maltose to maltopentaose, wherein nearly 1.5-times more activity was observed as compared to starch. For substrates like maltohexaose and maltoheptaose, the rate of initial hydrolysis reduced. As compared to glycogen, pullulan was hydrolysed slowly (5% activity). With carboxymethyl pullulan, a substrate relatively more specific for endo-hydrolysing enzymes, the initial rate of hydrolysis

Fig. 4.16: The rate of hydrolysis of pullulan and soluble starch by the *S. rolfsii* enzyme in 0.05 M acetate buffer (pH 4.9) at 50°C.



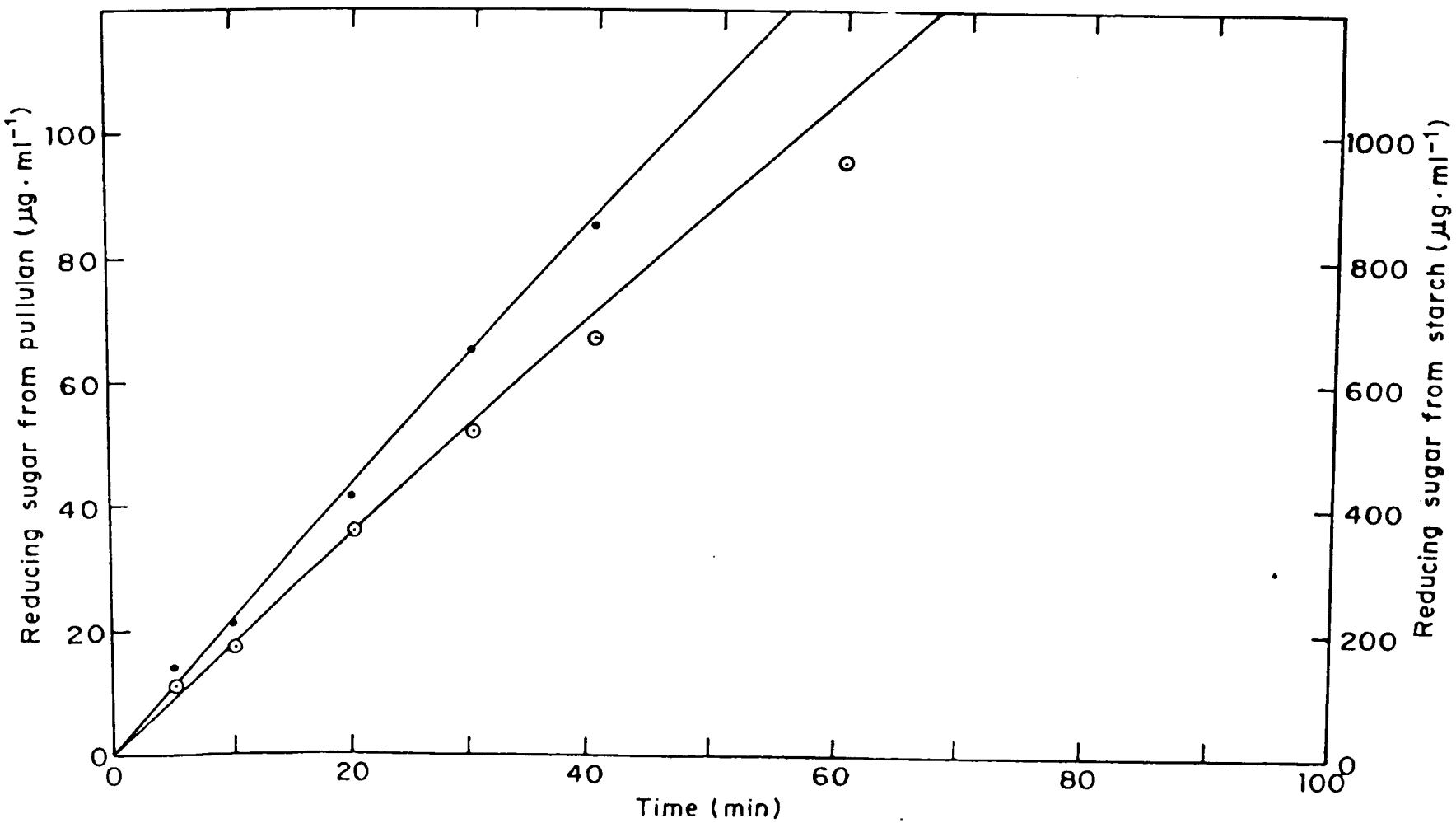

Reducing sugar from pullulan (μg · ml⁻¹)
Reducing sugar from starch (μg · ml⁻¹)
Time (min)
0
20
40
60
80
100
200
400
600
800
1000

Table 4.4 : Initial rates of hydrolysis of different substrates.

Substrate	Linkage type	Percent of maximum activity
Pullulan	α-(1->4)- and α-(1->6)-	5.0
Soluble starch	α-(1->4)- and α-(1->6)-	100.0
Amylose	α-(1->4)-	60.0
Amylopectin	α-(1->4)- and α-(1->6)-	67.0
Dextrin	α-(1->4)-	94.0
Potato starch (raw)	α-(1->4)- and α-(1->6)-	20.0
Glycogen	α-(1->4)- and α-(1->6)-	100.0
Maltose	α-(1->4)-	60.0
Maltotriose	α-(1->4)-	77.0
Maltotetraose	α-(1->4)-	107.0
Maltopentaose	α-(1->4)-	140.0
Maltohexaose	α-(1->4)-	90.0
Maltoheptaose	α-(1->4)-	67.0
Panose	α-(1->4)- and α-(1->6)-	23.3
Carboxymethyl pullulan	α-(1->4)- and α-(1->6)-	2.5
Isomaltotriose[a]	α-(1->4)- and α-(1->6)-	0.002
Dextran[a]	α-(1->6)-	0.008
Avicel (micro-cryst. cellulose)[b]	β-(1->4)-	N.D.
Chitin[b]	β-(1->4)-	N.D.
Xylan[b]	β-(1->4)-	N.D.

Note : Activities as described in section 4.2.6.10 (a)

a With ten-fold more enzyme in the reaction mixture

b N.D. not detected, even after using ten-times more enzyme concentration and an increased incubation period of upto 2 h.

was only of the order of 50% as compared to native pullulan. A very low activity was observed when dextran (a polyglucose polymer with α-(1->6)-linkages) was used as a substrate, with ten times more the enzyme concentration than used normally. With predominantly β-(1->4)-linked substrates like Avicel (micro-crystalline cellulose), chitin and xylan, no activity was observed.

b. *Products of hydrolysis of the various α-D-glucans as a function of time*

Paper chromatographic examination of the reaction products from various α-D-glucans (pullulan, soluble starch, amylose, amylopectin, glycogen) as a function of time clearly indicated presence of glucose as the sole product of hydrolysis. This confirms the exo-hydrolytic action of the enzyme on the various α-D-glucans tested (Fig. 4.17 a-e).

4.2.6.11 Inhibition studies with acarbose

Acarbose (an analog of maltotetraose) produced by a *Actinoplanes* sp. is reported to be a strong competitive inhibitor of α-D-glucosidases (Schmidt *et al.*, 1977). It has also been used for affinity purification of glucoamylases from various fungi (Ono and Smith, 1986; DeMot and Verachtert, 1987; Ono *et al.*, 1988a,b,c). Since, the enzyme from *S. rolfsii* showed pullulan-, as well as starch-hydrolysing activities, inhibition studies were carried out using acarbose.

The initial studies indicated that acarbose caused a strong inhibition of both of the enzyme activities. The Michaelis-Menten treatment indicated the mode of action of the inhibitor as competitive for both starch-, as well as, pullulan-hydrolysis. The inhibitor constant (K_i) determined for the inhibition of pullulan-hydrolysis was 4.0×10^{-3} μM and for the hydrolysis of starch was 6.0×10^{-2} μM (Fig. 4.18 a,b,c).

Fig. 4.17 a,b: Paper chromatography of the products of hydrolysis of (a) pullulan and (b) soluble starch by the *S. rolfsii* enzyme (20 μg). The time course hydrolysis of the respective substrate (10 mg) was carried out and the hydrolysate aliquots were applied at intervals to Whatman Paper No.1 and chromatographed in butanol:pyridine:water (6:4:3 v/v) with two ascents. The sugars on the paper were visualized by the alkaline silver nitrate method of Trevelyan *et al.* (1950) modified by Robyt and French (1963).

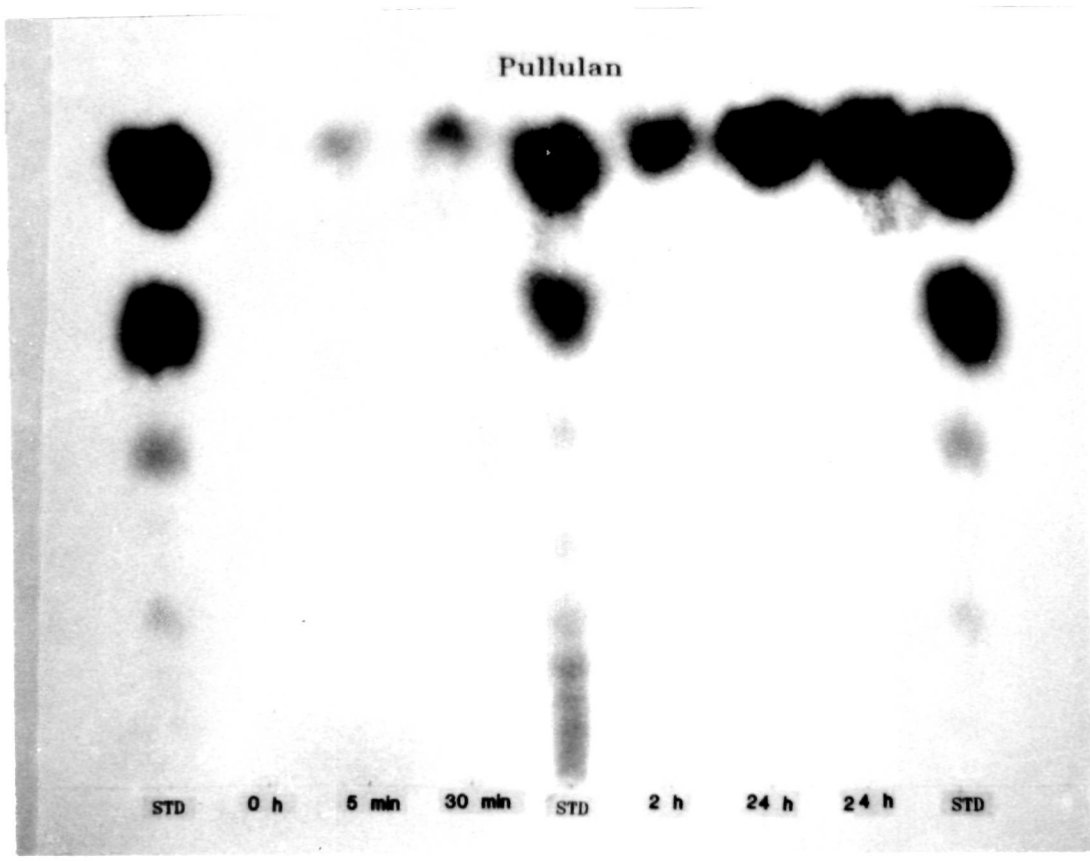


Fig. 4.17 a

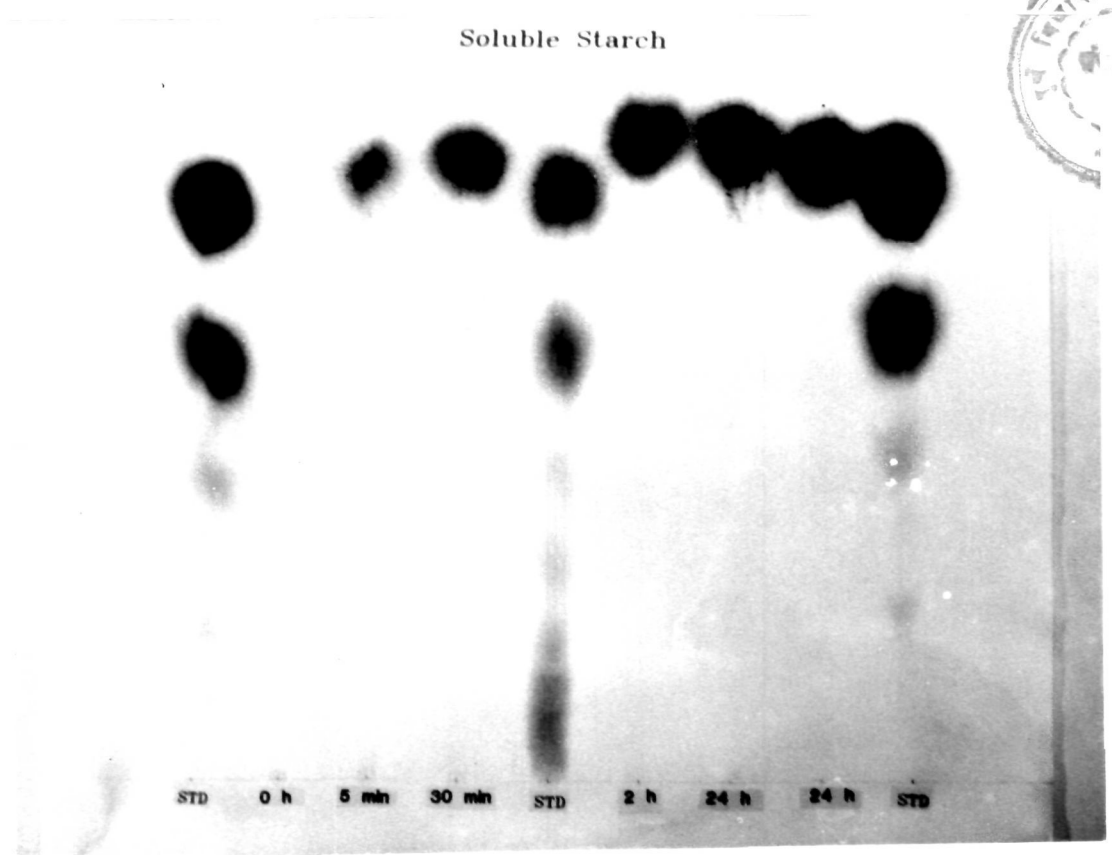


Fig. 4.17 b

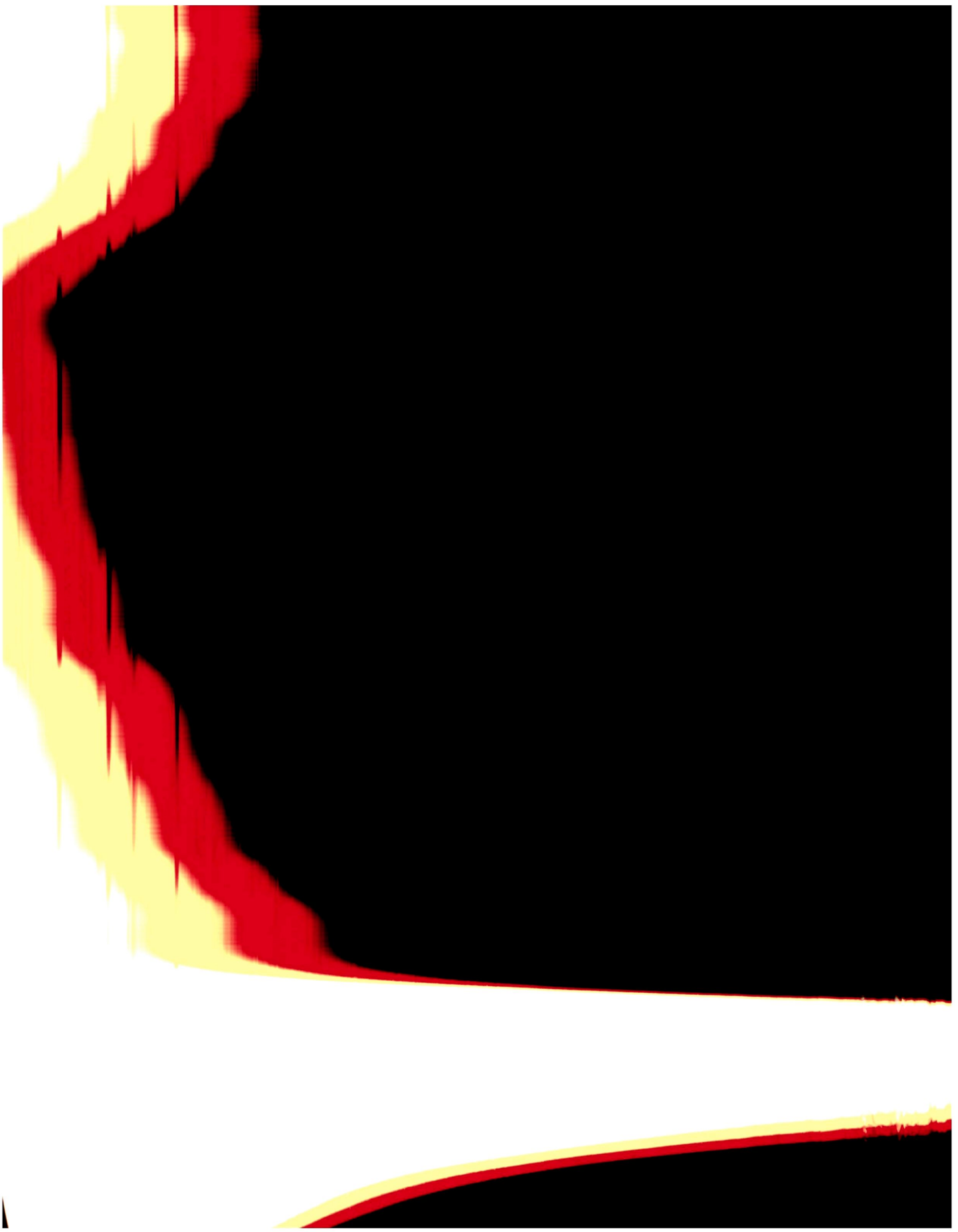


Fig. 4.17 c,d: Paper chromatography of the products of hydrolysis of (c) amylopectin and (d) glycogen by the *S. rolfsii* enzyme (20 μg). The time course hydrolysis of the respective substrate (10 mg) was carried out and the hydrolysate aliquots were applied at intervals to Whatman Paper No.1 and chromatographed in butanol:pyridine:water (6:4:3 v/v) with two ascents. The sugars on the paper were visualized by the alkaline silver nitrate method of Trevelyan *et al.* (1950) modified by Robyt and French (1963).

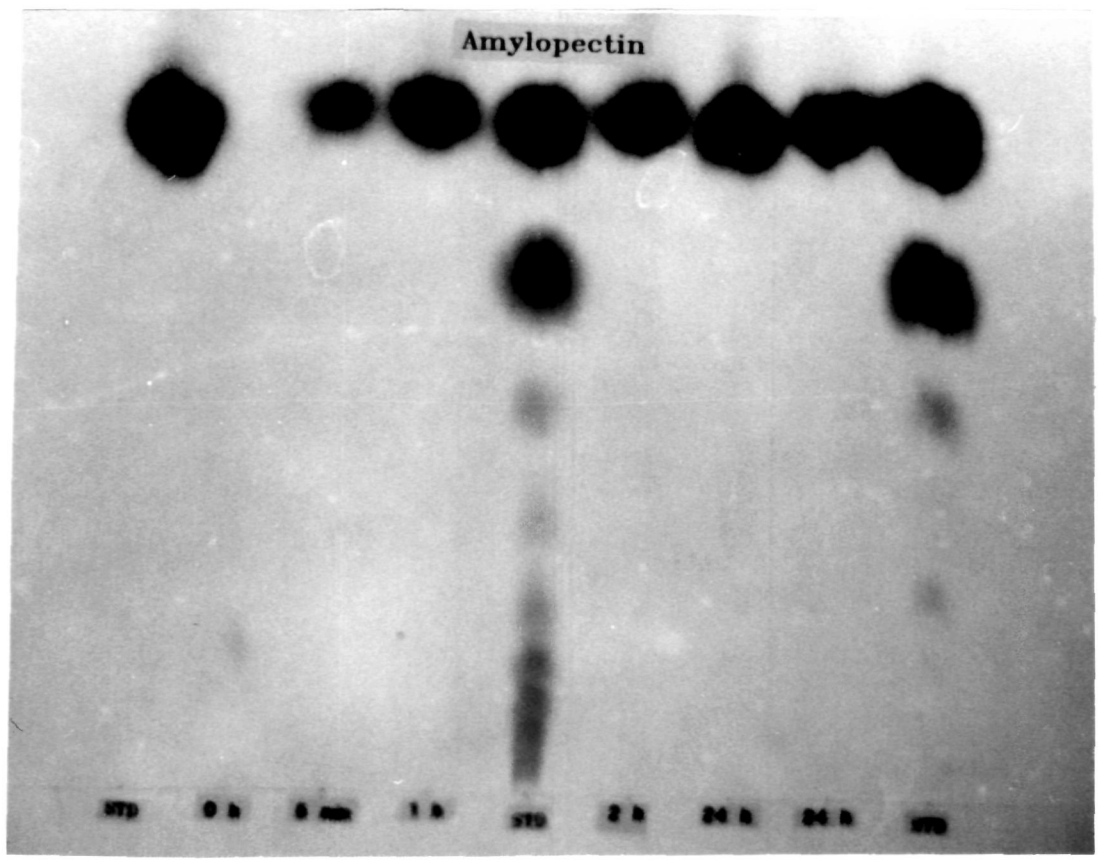


Fig. 4.17 c

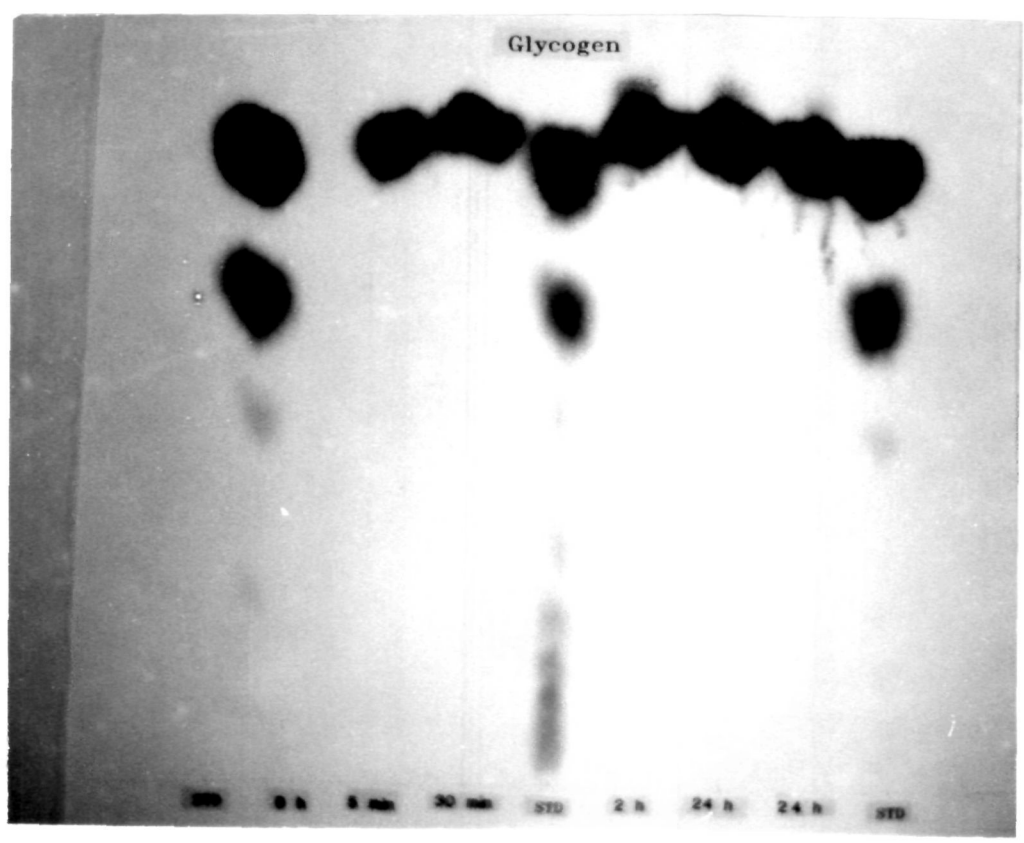


Fig. 4.17 d

Fig. 4.17 e : Paper chromatography of the products of hydrolysis of (e) amylose by the *S. rolfsii* enzyme (20 μg). The time course hydrolysis of the respective substrate (10 mg) was carried out and the hydrolysate aliquots were applied at intervals to Whatman Paper No.1 and chromatographed in butanol:pyridine:water (6:4:3 v/v) with two ascents. The sugars on the paper were visualized by the alkaline silver nitrate method of Trevelyan *et al.* (1950) modified by Robyt and French (1963).

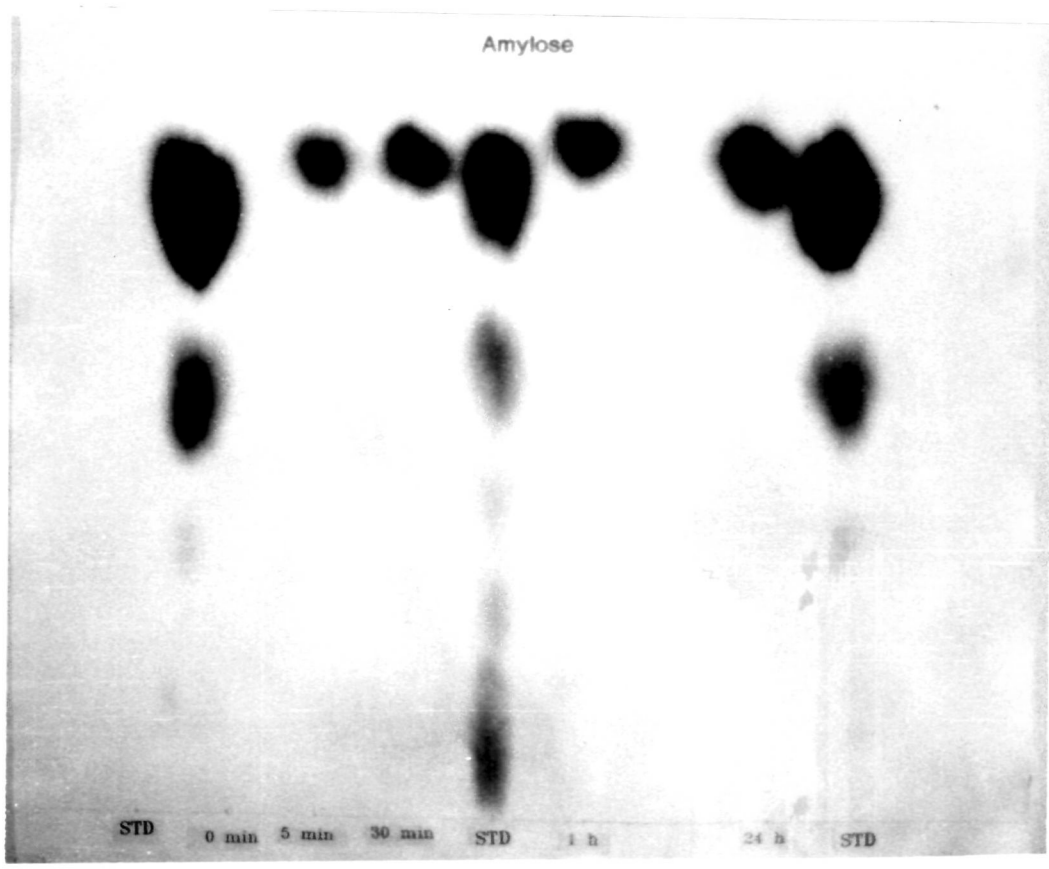


Fig. 4.17 e

Fig. 4.18 a: Lineweaver-Burk plot for the determination of the effect of varying concentrations of acarbose on the pullulan-hydrolysis by the *S. rolfsii* enzyme. The enzyme was incubated with different concentrations of pullulan (Sigma Chemical Co, U.S.A.) at 50°C in 0.05 M acetate buffer (pH 4.9), with a fixed concentration of acarbose. The reducing sugars released were estimated by the Somogyi-Nelson method (1952).

(●——●) Control

(△——△) 10 $ng.ml^{-1}$

(○——○) 20 $ng.ml^{-1}$

(⊙——⊙) 50 $ng.ml^{-1}$

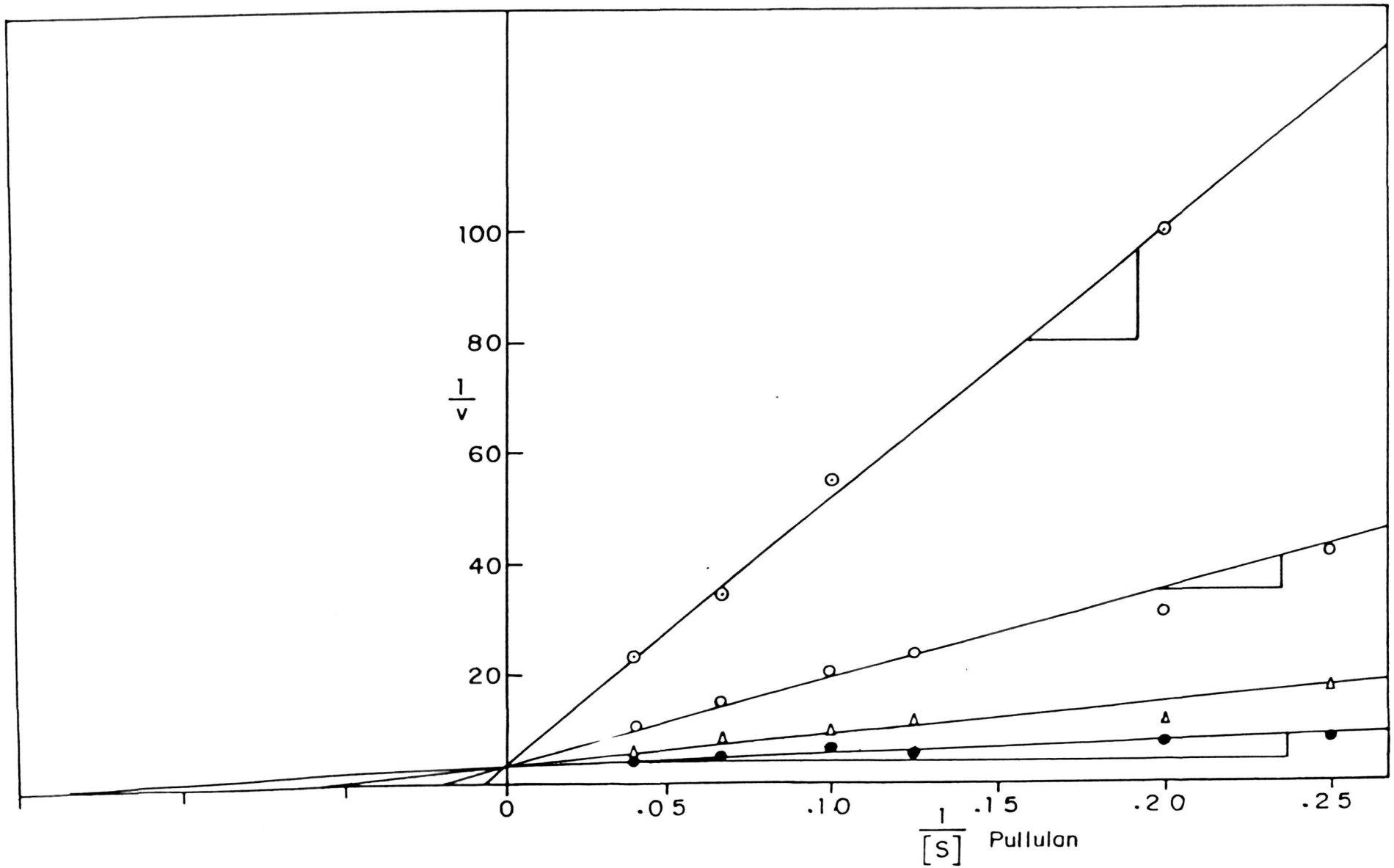

100
80
1/v
60
40
20
0
.05
.10
.15
.20
.25
1/[S] Pullulan

Fig. 4.18 b: Lineweaver-Burk plot for the determination of the effect of varying concentrations of acarbose on the soluble starch-hydrolysis by the *S. rolfsii* enzyme. The enzyme was incubated with different concentrations of soluble starch (Connought Medical Laboratories, Canada) at 50°C in 0.05 M acetate buffer (pH 4.9), with a fixed concentration of acarbose. The reducing sugars released were estimated by the DNS method (Fischer and Stein, 1961).

(●——●) Control

(○——○) 25 ng.ml^{-1}

(⊗——⊗) 50 ng.ml^{-1}

(⊡——⊡) 100 ng.ml^{-1}

(△——△) 250 ng.ml^{-1}

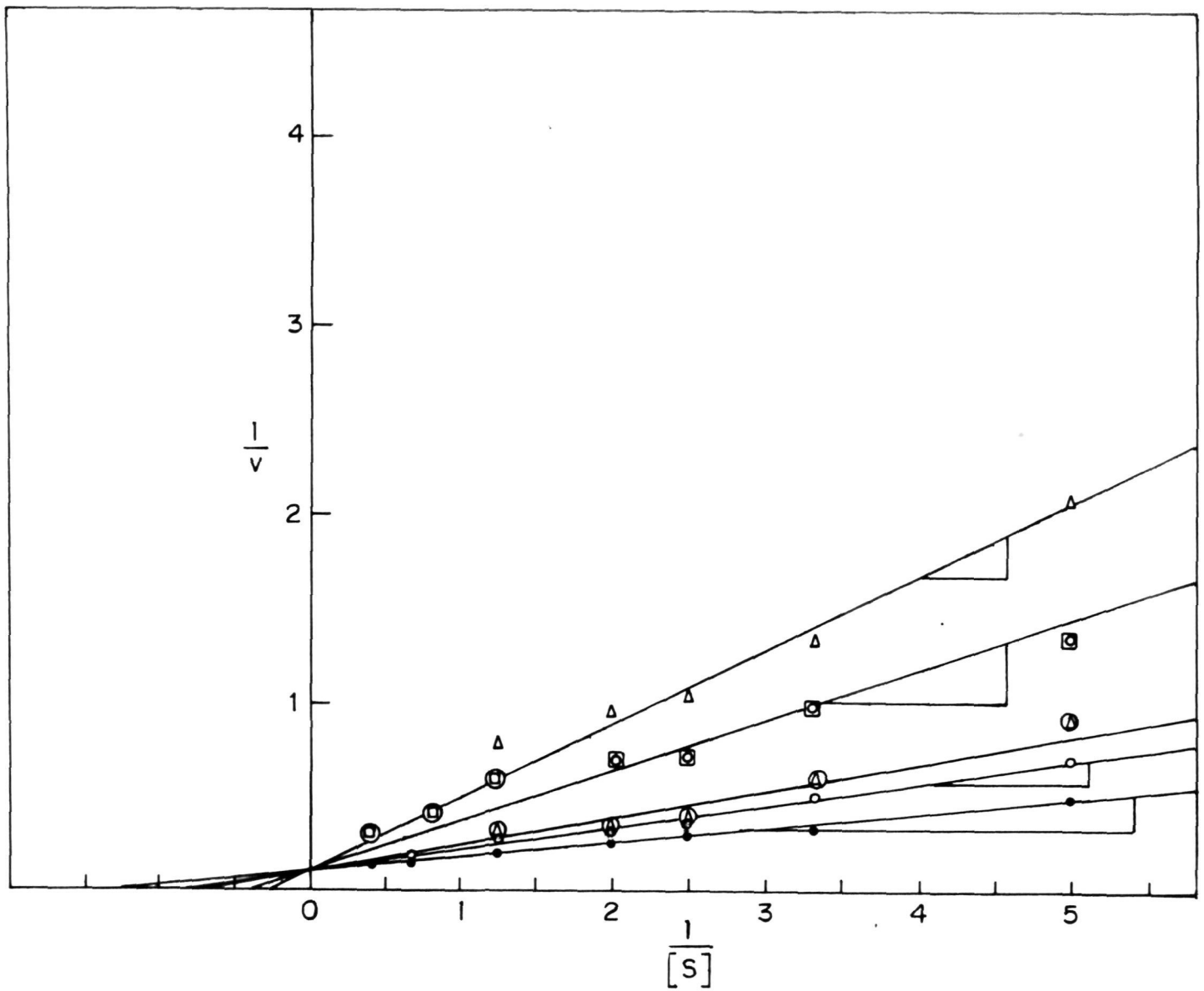

4
3
2
1
0
1
2
3
4
5
1/v
1/[S]

Fig. 4.18 c: The slopes of the lines obtained in both of the cases were plotted against the respective inhibitor concentration to obtain an estimate of the inhibitor constant (K_i) for acarbose for the hydrolysis of both of the substrates by the *S. rolfsii* enzyme.

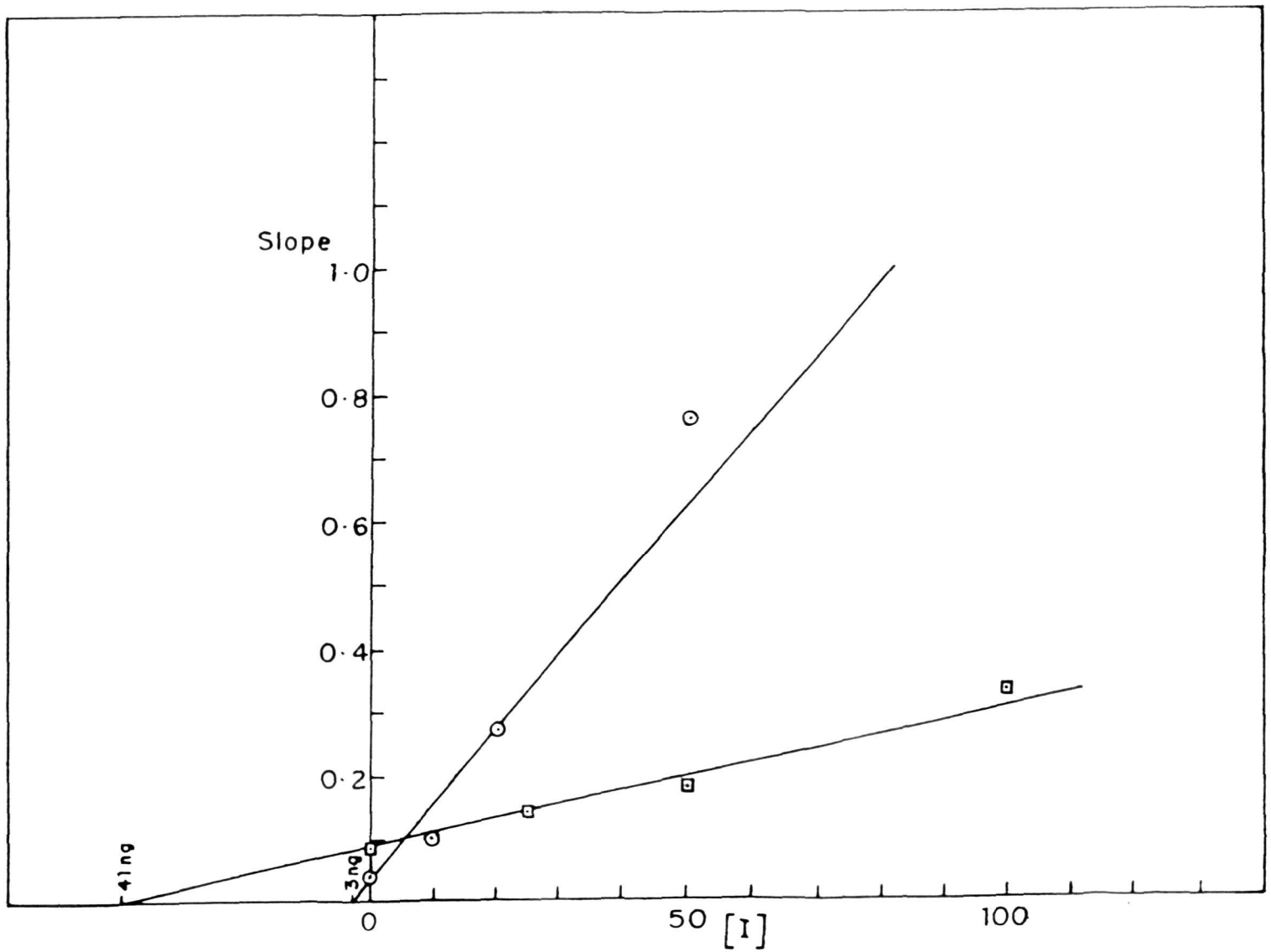
(⊙——⊙) Pullulan

(⊡——⊡) Soluble-starch

Fig. 4.18 c: The slopes of the lines obtained in both of the cases were plotted against the respective inhibitor concentration to obtain an estimate of the inhibitor constant (K_i) for acarbose for the hydrolysis of both of the substrates by the *S. rolfsii* enzyme.

(⊙——⊙) Pullulan

(⊡——⊡) Soluble-starch


Slope
1·0
0·8
0·6
0·4
0·2
0
50
[I]
100
4 ng
3 ng

A comparison of the inhibition of the enzyme activity on pullulan by inclusion of acarbose in the reaction mixture was carried out. It can be seen that the inclusion of acarbose (50 μg) in the reaction mixture completely inhibited the hydrolysis of pullulan, as compared to the respective controls (Fig.4.19).

4.3 DISCUSSION

The pullulan-hydrolysing enzyme was purified from the supernatants of a 10 days old culture of *S. rolfsii* (NCIM 1084) grown in NM-2 medium containing soluble starch (1% w/v) as the carbon source. During the initial purification studies, it was observed that the pullulan-hydrolysing activity was always accompanied by the starch-hydrolysing activity. Therefore, efforts were made to resolve, these two enzyme activities. For this, preparative isoelectric focusing (pH 3.5 - 10.0 and 2.5 - 6.0), hydrophobic interaction chromatography (Phenyl-Sepharose CL-6B) and affinity chromatography on Concanavalin A-Sepharose CL-4B were tried in addition to the procedures described in the purification schemes. However, in all of these procedures, both the hydrolytic activities appeared together. Similar observations were recorded for the pullulan-hydrolysing glucoamylase from *Candida antarctica* CBS 6638 by DeMot and Verachtert (1987), who reported the routine use of pullulan-hydrolysis to monitor the elution of this glucoamylase during the chromatographic procedures. This avoided the specific detection of glucose by the enzymatic method and allowed them to carry out the activity estimations without dilution of the enzyme samples.

The molecular weight of the enzyme was determined to be about 64,000 daltons, which is comparable to the values reported for the pullulan-hydrolysing enzymes from other sources (section 1.3.3.1). The enzyme protein apparently consisted of one polypeptide chain with absence of a sub-unit structure.

Fig. 4.19 a,b: Effect of addition of acarbose (50 μg) on the hydrolysis of pullulan by the *S. rolfsii* enzyme (reaction carried out at 25°C in 0.05 M acetate buffer, pH 4.9).

(a) Control without acarbose

(1) standard

(2) blank (0 h)

(3) products of hydrolysis at the end of 1 h by the *S. rolfsii* enzyme

(4) products of hydrolysis at the end of 1 h by the *Klebsiella pneumoniae* pullulanase (Sigma Chemical Company, U.S.A.)

(5) products of hydrolysis at the end of 1 h by a combination of *S. rolfsii* enzyme and the pullulanase from *K. pneumoniae*

(b) With acarbose

(1) Standard

(2) blank containing acarbose (50 μg)

(3) products of hydrolysis at the end of 1 h by the *S. rolfsii* enzyme in presence of acarbose (50 μg)

(4) products of hydrolysis at the end of 1 h by the *K. pneumoniae* pullulanase in presence of acarbose (50 μg)

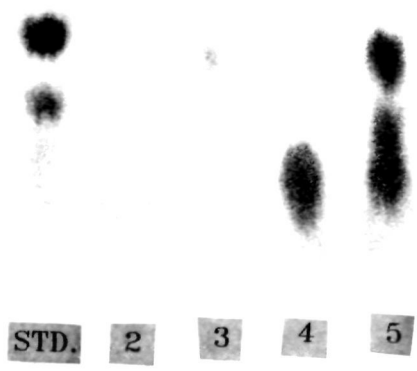


Fig. 4.19 a

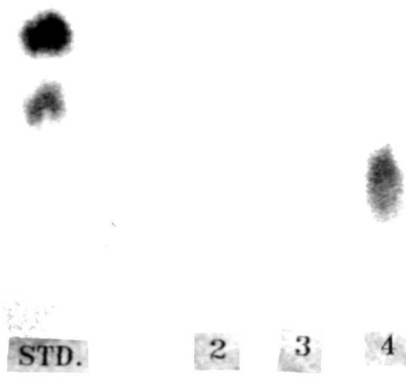


Fig. 4.19 b

The enzyme was found to contain 12 - 13% carbohydrate (by weight) as determined by the phenol - H_2SO_4 method indicating that some glycosyl residues were present on the protein. The enzyme showed a strong affinity towards Concanavalin A. Strong interaction of the enzyme with Concanavalin A and its specific elution with α-methyl-D-mannopyranoside indicated that the glycosyl side chains associated with the enzyme could probably be rich in mannose residues. Glycoprotein staining with the use of thymol-H_2SO_4 staining procedure further confirmed the association of carbohydrate with the enzyme protein. In general, the literature survey showed that there is a paucity of information regarding the side-chain modifications present in the pullulan-hydrolysing enzymes known till today. A significant exception to this observation has been the pullulanase from *K. pneumoniae*, which has been recently shown to be a lipoprotein (Pugsley *et al.*, 1986).

In keeping with the general trend observed with the pullulan-hydrolysing enzymes, the enzyme was observed to be capable of hydrolysis of pullulan and starch in an overlapping pH range of between 4.0 - 4.5. In this range, almost similar relative rates for the hydrolysis of starch and pullulan were obtained (Fig. 4.7, 4.8). This observation can prove to be of practical significance because, most of the currently used starch processing is done in the acidic pH range of 4.5 - 6.5 (Kennedy *et al.*, 1988).

The temperature optima of the enzyme for the hydrolysis of pullulan (50°C) and starch (65°C) were different. Similar observations were reported by McCleary and Anderson (1980) for the pullulan-hydrolysing glucoamylase from *Cladosporium resinae*. They attributed the difference in the temperature optima to the relative affinities of the enzyme for the substrates. It was postulated that in case of starch, the

relative predominance of the non-reducing end groups stabilizes the enzyme (more enzyme can be bound to the substrate at any time) against temperature. A similar mechanism may also be advocated for the enzyme in the present studies.

The thermostability of the enzyme with respect to both of the substrates was found to be almost identical (Fig. 4.15). The enzyme activities were almost stable (90%) for 15 min upto 50°C. Temperatures of more than 80°C were observed to inactivate both the enzyme activities in 15 min. The identical thermal inactivation characteristics of the analogous activities in case of the glucoamylase P of *C. resinae* have been considered to rule out the possibility of presence of two enzymes in their preparation by Fagerström *et al.* (1990). A similar explanation may be applied to the enzyme in the present studies.

Since the enzyme in the present study showed an hydrolytic action against both pullulan and starch, it was of interest to explore the substrate specificity and the mode of action of the enzyme. Out of the α -, ß-D-glucans and oligosaccharides tested, the enzyme showed an absolute specificity for the sugars linked in the α -configuration. The enzyme did not have any hydrolytic action against ß-linked substrates such as micro-crystalline cellulose, chitin and xylan, even in a large excess of the enzyme or with extended times of incubation. The enzyme exhibited a rapid hydrolysis of α -(1->4)-glucosidic linkages with the polymers being hydrolysed more efficiently. Since, the initial rates of hydrolysis of the series of maltooligosaccharides went on increasing till maltopentaose (DP5), the enzyme appeared to be an exo-glucanase and not an α -glucosidase (Reese *et al.*, 1968).

Pullulan was hydrolysed at about 5% the rate of initial hydrolysis observed for the soluble starch. In this respect, the enzyme appeared to resemble the

pullulan-hydrolysing glucoamylase "S" described in the case of *C. resinae* by McCleary and Anderson (1980). The enzyme also showed a significant (20%) rate of hydrolysis of raw potato starch. This is an important observation, since the use of raw starch hydrolysing enzyme for the cold-saccharification of starch is being advocated for certain applications (Kennedy *et al.*, 1988; Saha and Zeikus, 1989).

Since, the pullulan-hydrolysing enzyme from *S. rolfsii* showed hydrolysis of pullulan- as well as starch, inhibition studies were carried out using acarbose to check for the presence of a contaminating activity capable of endo-hydrolysis of pullulan at the α-(1->6)-linkages. It appeared from the chromatography of the reaction products that there was no contaminating α-(1->6)-hydrolysing activiy present.

The paper chromatographic analysis of the products indicated that glucose was the sole product of hydrolysis of the substrates tested. The rapid appearance of glucose (within 5 min) indicated the exo-hydrolysis of these substrates, including pullulan. Our observations thus strongly suggest that the purified enzyme from is a 1,4- α -D-glucan glucohydrolase (glucoamylase, EC 3.2.1.3), which shows a significant action on pullulan.

CHAPTER V

5.1 INTRODUCTION

Over the past decade, the role of fungal protoplasts in a variety of biochemical and physiological investigations has gained considerable importance. Protoplasts have been used as an effective experimental tool for studying the cell wall synthesis (Necas and Svoboda, 1985), enzyme secretion (Kolar *et al.*, 1985; Deshpande *et al.*, 1987; Johri *et al.*, 1990) and steroid transformation (Dlugonski *et al.*, 1984). Lately the fungal protoplast fusion and transformation systems have been developed as an aid to further our understanding of some fundamental phenomenon such as genetic incompatibility between strains and species (Kawasumi *et al.*, 1988; Stasz *et al.*, 1989; Toyama and Toyama, 1990; Ushijima *et al.*, 1990). Recently, protoplasts have also been used as single cell preparations for mutagenesis protocols towards strain improvement (Mukherjee and Sengupta, 1986; Homolka, 1988; Junwei and Shuyun, 1988; Hebraud and Fevre, 1988). For the successful implementation of any of these studies, standardization of a rapid method for the release of viable protoplasts becomes an essential prerequisite.

Though *S. rolfsii* has been studied extensively for cellulolytic enzymes (Lachke and Deshpande, 1988), the inability of the organism to produce asexual spores and the mycelial clump formation during normal growth have hampered its biotechnological applications. Protoplasts of *S. rolfsii* offer a convenient system that can be used to overcome some of these difficulties. Though the successful isolation of protoplasts of *S. rolfsii* was reported (Deshpande *et al.*, 1987), the time required for the isolation of the protoplasts was long and the viability of the protoplasts in terms of the regeneration frequency was low (0.6%). *S. rolfsii* is known to produce all the

three amylolytic activities, namely, α-amylase, glucoamylase (Takao *et al.*, 1986) and a pullulanase-type of activity required for the hydrolysis of starch (Kelkar *et al.*, 1988). Hence, the present investigation were undertaken, (a) to develop a rapid procedure for the isolation of viable *S. rolfsii* protoplasts, (b) to immobilize these protoplasts in calcium alginate gels to evaluate their potential for starch hydrolysis.

5.2 RESULTS

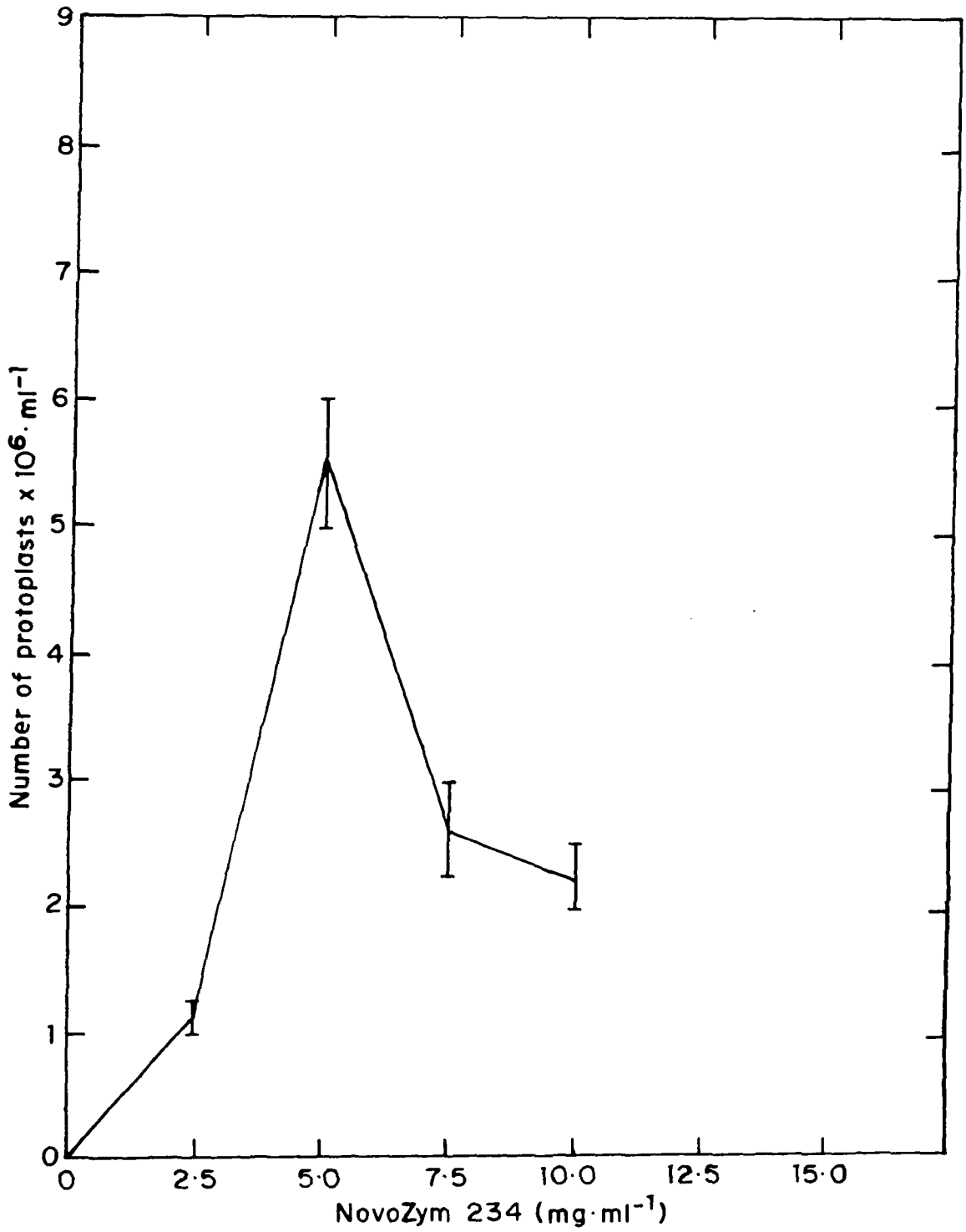
During the initial experiments, it was observed that a rapid release of protoplasts occurred when *S. rolfsii* mycelium was incubated in 0.05 M maleic acid - NaOH buffer (pH 5.0) stabilized with 0.7 M NaCl containing NovoZym 234 (5 $mg.ml^{-1}$). Hence, further optimization studies were carried out using NovoZym 234 as the mycolysing enzyme preparation.

5.2.1 Factors affecting the release of protoplasts in S. rolfsii

5.2.1.1 Effect of NovoZym 234 concentration

Different concentrations of NovoZym 234 (2.5 - 10 $mg.ml^{-1}$) were subsequently tried to check the influence on the isolation of protoplasts from *S. rolfsii*. The results of these experiments are summarized in Fig. 5.1. A 5 $mg.ml^{-1}$ concentration of NovoZym 234 as found to give the highest yields of protoplasts (5 - 6 X 10^6 $.ml^{-1}$). At higher concentrations, the enzyme adversely affected the released protoplasts causing nearly a 50% lysis (2 - 3 X $10^6.ml^{-1}$). Concentrations of NovoZym 234 less than 5 $mg.ml^{-1}$ resulted in a decrease in the yield of protoplasts obtained, when compared with the NovoZym 234 at a concentration of 5 $mg.ml^{-1}$.

Fig. 5.1: Effect of concentration of NovoZym 234 on the release of protoplasts of *S. rolfsii* (section 5.2.1.1). Bar markers represent standard variation from the mean values.


Number of protoplasts x 10^6. ml^{-1}
0
1
2
3
4
5
6
7
8
9
0
2·5
5·0
7·5
10·0
12·5
15·0
NovoZym 234 (mg·ml^{-1})

5.2.1.2 Selection of the osmotic stabilizer

Results of experiments using different inorganic salts, sugars and sugar alcohols as potential stabilizers of protoplasts of *S. rolfsii*, are summarized in Table 5.1. Keeping with the general trend observed for the filamentous fungi, it was noted that the inorganic salts were efficient in stabilizing the protoplasts of *S. rolfsii*. Out of the inorganic salts tested KCl (0.6 M, 5 - 6 X 10^6.ml^{-1}) and NaCl (0.7 M, 4 - 5 X 10^6.ml^{-1}) were found to offer the best protection. Protoplasts isolated in NaCl and KCl were observed to be vacuolated in the later stages (4 h) of isolation. Freshly isolated protoplasts were always found to be small and predominantly non-vacuolated. Protoplasts obtained in the presence of $MgSO_4$ (0.6 M) and $MgCl_2$ (0.7 M) were found to be fragile and tended to lyse. This was reflected in the reduced yields (2 - 3 X 10^6.ml^{-1}) that were finally obtained in 4 h.

5.2.1.3 Effect of incubation time

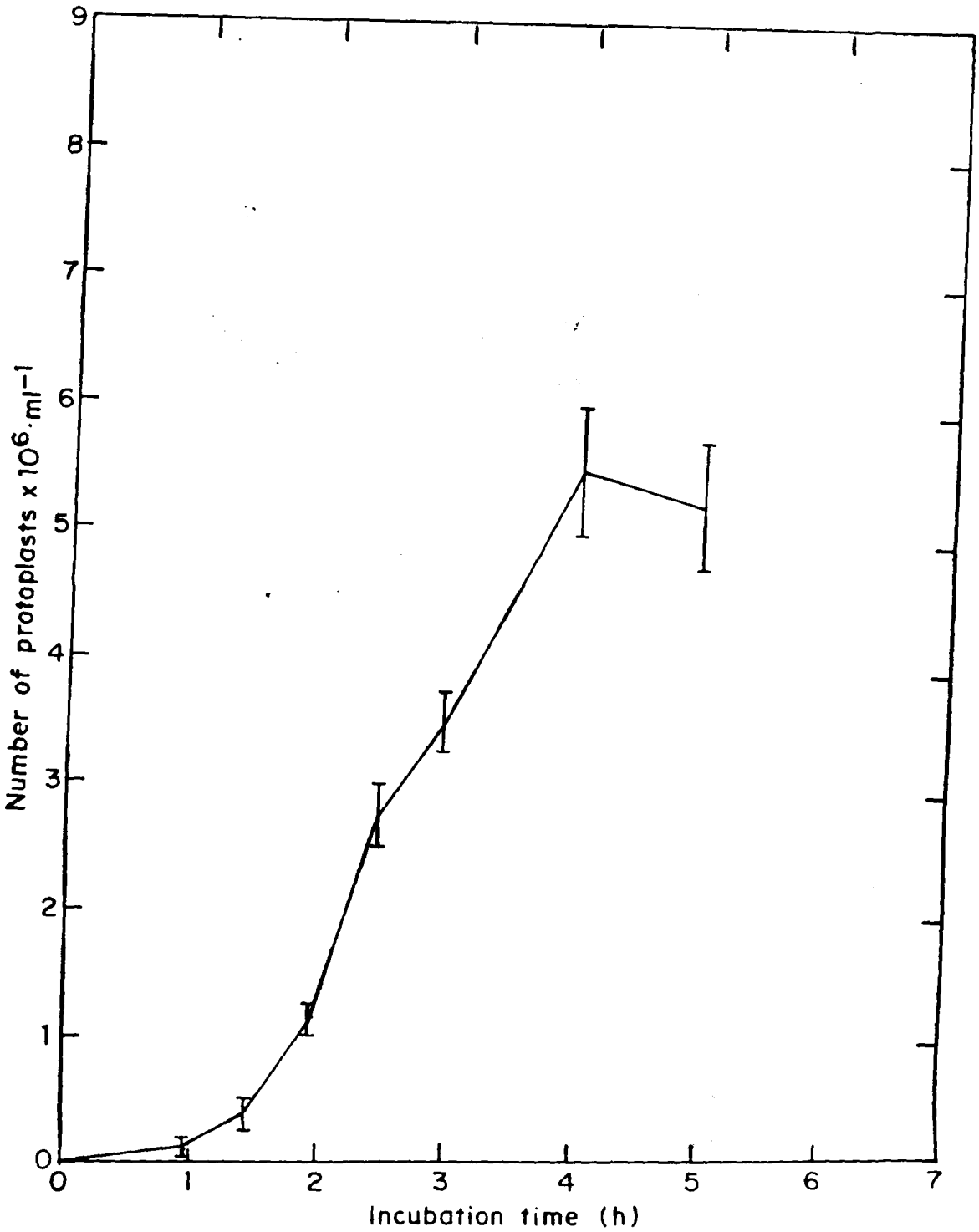
The release of protoplasts in the supernatant fluid of the reaction mixture became evident very early in the reaction (30 min). The degradation of the mycelial clump into small fragments was apparent visually. Countable numbers of protoplasts were seen to be released typically by the end of first hour of lytic digestion. A typical experiment when followed sequentially as a function of time has been reproduced in Fig. 5.2. It can be seen that the maximum release of the protoplasts occurred between the second and fourth hour of digestion, which attained a saturation by the end of four hours. Further increasing the time of incubation, resulted in a slight decrease in the number of protoplasts. The reaction mixture at the end of four hours appeared to be in the form of a uniform suspension. The release of the protoplasts was confirmed to be predominantly sub-apical in origin, by direct microscopic observations of cultures

Table 5.1 : Effect of various osmotic stabilizers on isolation of *Sclerotium rolfsii* protoplasts.

Osmotic stabilizer	Molarity (M)	Number of protoplasts[a] (X 10^6 ml^{-1})
NaCl	0.7	4.5 ± 0.5
KCl	0.5	3.0 ± 0.2
	0.6	5.5 ± 0.6
	0.7	4.0 ± 0.5
$MgSO_4$	0.6	2.2 ± 0.3
$MgCl_2$	0.7	2.5 ± 0.5
NH_4Cl	0.4	1.8 ± 0.2
Sorbitol	0.4	0.8 ± 0.2
Sucrose	0.6	-

a In 0.05 M maleic acid - NaOH buffer (pH 5.0) with NovoZym 234 (5 $mg.ml^{-1}$) at 30°C in 4 h.

Fig. 5.2: Release of protoplasts as a function of time in case of *S. rolfsii* under standard conditions (section 5.2.1.3). Bar markers represent standard variation from the mean values.


Number of protoplasts x 10^6.ml^{-1}
Incubation time (h)
0
1
2
3
4
5
6
7
8
9

grown on cover slips.

5.2.1.4 Age of mycelium

The ability of NovoZym 234 to release protoplasts from the mycelia grown for different periods of time (24 - 144 h) was tested (Fig. 5.3). The best results with respect to time and the yield of protoplasts were obtained when 24 h old mycelium was subjected to mycolysis with NovoZym 234. An incubation time of minimum of 24 h is required to obtain appreciable growth of *S. rolfsii* in MYG medium. With increase in the time of culture, there was a sharp decrease in the yield of protoplasts, indicating a build-up of resistance to the lytic digestion by NovoZym 234 in older cultures of *S. rolfsii* (Fig. 5.3).

5.2.1.5 Amount of mycelium

The amount of mycelium (24 h) old supporting the maximum yield of protoplasts was investigated using NovoZym 234 (5 $mg.ml^{-1}$) (Fig. 5.4). There was a steady increase in the yield of the protoplasts with an increasing amount of the mycelium from 10 $mg.ml^{-1}$ to 50 $mg.ml^{-1}$ (damp weight). Increasing the amount of the mycelium further did not give an increase in the efficiency of protoplast release.

5.2.1.6 Pretreatment with thiol compounds

Dithiothreitol (5 mM) and 2-mercaptoethanol (1 $\mu l.ml^{-1}$) were tried singly and in combination with EDTA (50 mM) for the pretreatment of the *S. rolfsii* mycelium prior to the enzymatic digestion. There was no appreciable change in the number of protoplasts released or in the time required for the release of the protoplasts.

Fig. 5.3: Protoplast release in relation to mycelial age in case of *S. rolfsii* under standard conditions (section 5.2.1.4). Bar markers represent standard variation from the mean values.

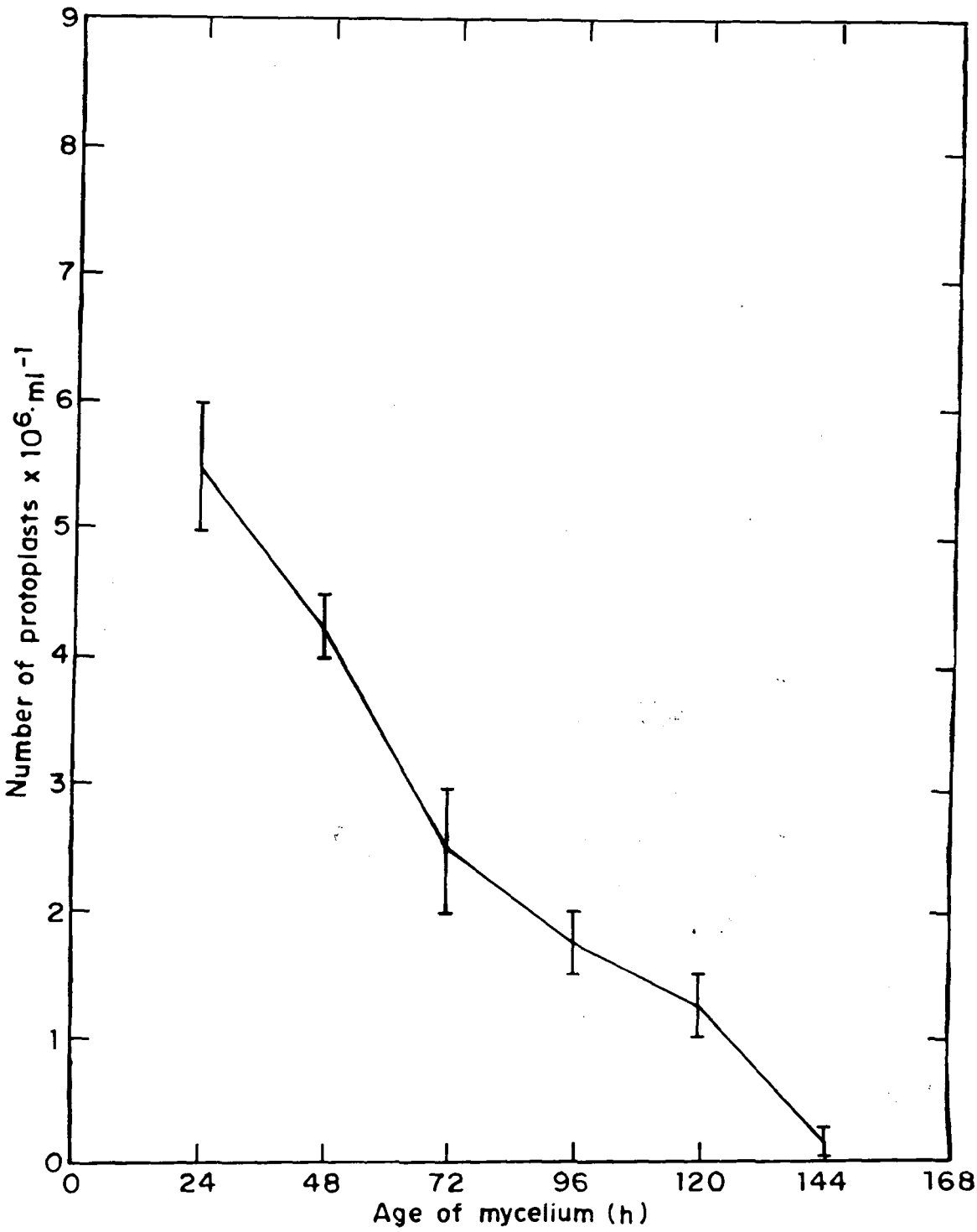
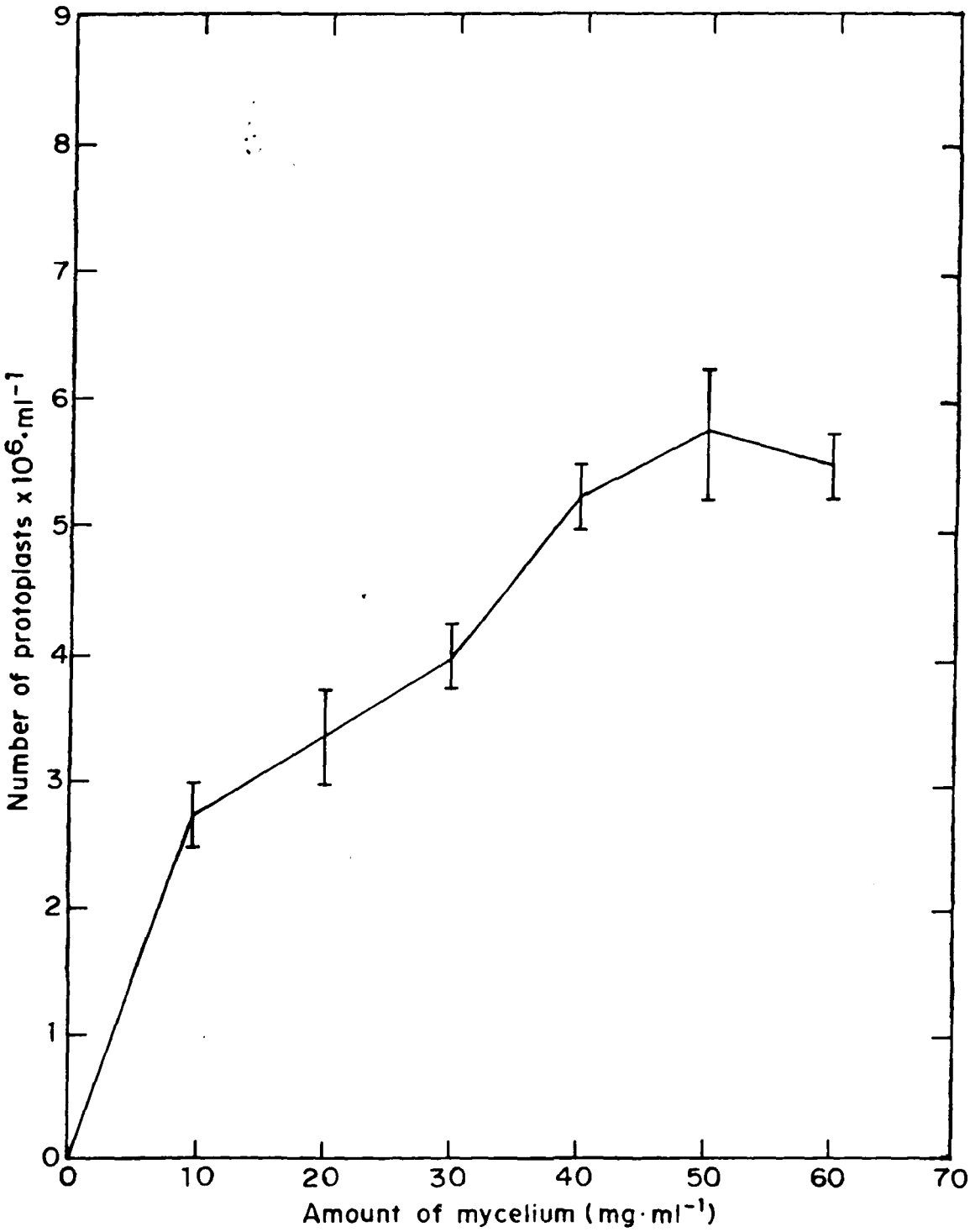

Number of protoplasts x 10^6.ml^{-1}
0
1
2
3
4
5
6
7
8
9
0
24
48
72
96
120
144
168
Age of mycelium (h)

Fig. 5.4: Protoplast release in relation to amount of the mycelium under standard conditions (section 5.2.1.5). Bar markers represent standard variation from the mean values.


Number of protoplasts x 10^6.ml^{-1}
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
Amount of mycelium ($mg \cdot ml^{-1}$)

5.2.2 Factors affecting the regeneration of protoplasts

5.2.2.1 Effect of osmotic stabilizers

Regeneration of the protoplasts was followed by the thin layer agar (TLA) method of Kobayashi *et al.* (1985). Though KCl (0.6 M) was found to be the best osmotic stabilizer for the isolation of protoplasts of *S. rolfsii*, it did not support efficient regeneration of the protoplasts (Table 5.2). Sucrose (0.6 M) and sorbitol (0.4 M) were found to be the best stabilizers supporting the optimum regeneration of the protoplasts of *S. rolfsii* (Table 5.2).

When the regeneration was followed using the TLA method, nearly 90 - 95% of the protoplasts were seen to be regenerating, with sucrose (0.6 M) as the osmotic stabilizer in the MYG medium. The actual values for the regeneration frequencies, based on the plate counts of the regenerated colonies were slighty lower (75 - 80%) (Table 5.2). Regeneration of the cell wall occurred on solid media within 5 - 6 h with the protoplast becoming insensitive to the osmotic shock by dilution. In case of the liquid media this lag was found to be of the order of 8 - 12 h. The protoplasts produced either one or two germ tubes, which branched out extensively forming a micro-colony that could be seen on the slide in 24 h (Fig. 5.5 a-e). Appearence of the visible colonies on plates required a period of 4 days of incubation at 30°C.

5.2.2.2 Effect of medium hardness

In the present studies, regenerating protoplasts of *S. rolfsii* predominantly showed the formation of germ tube after the completion of the cell wall regeneration. A small fraction (0.1%) of the regenerating protoplasts showed formation of irregularly shaped (budding-type) structures. These, at a later stage reverted back to normal

Table 5.2 Effect of various osmotic stabilizers on regeneration frequency of *Sclerotium rolfsii* protoplasts[a].

Osmotic stabilizer	Molarity (M)	Regeneration Frequency (%)[b]
NaCl	**0.7**	4 ± 2
KCl	**0.6**	3 ± 1
$MgSO_4$	**0.6**	3 ± 1
$MgCl_2$	**0.7**	6 ± 2
NH_4Cl	**0.4**	28 ± 2
Sorbitol	**0.4**	23 ± 2
Sucrose	0.6	90 ± 5

a In MYG medium with Difco Agar (2% w/v) with the stabilizer indicated at 30°C, 24 h.

b Direct microscopic observation using TLA method described by Kobayashi *et al.* (1985).

Fig. 5.5: Photomicrography of regeneration of *S. rolfsii* protoplasts. (a) 0 h; (b) 8 h; (c) 24 h; (d) anisodiametric regeneration; (e) light photomicrograph of a regenerating protoplast and (f) fluorescence photomicrograph of the protoplast in (e) stained with flourescein isothiocynate-wheat germ agglutinin for the specific detection of cell wall chitin. (X 500).

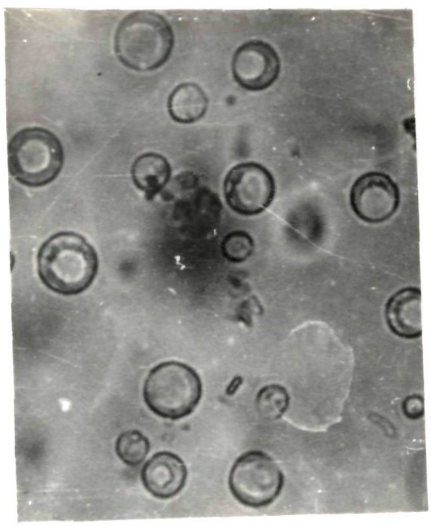


Fig. 5.5 a

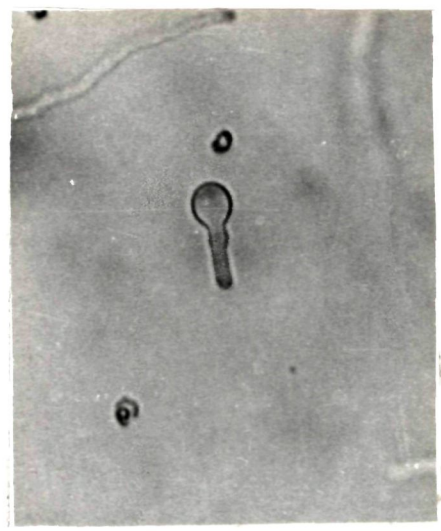


Fig. 5.5 b

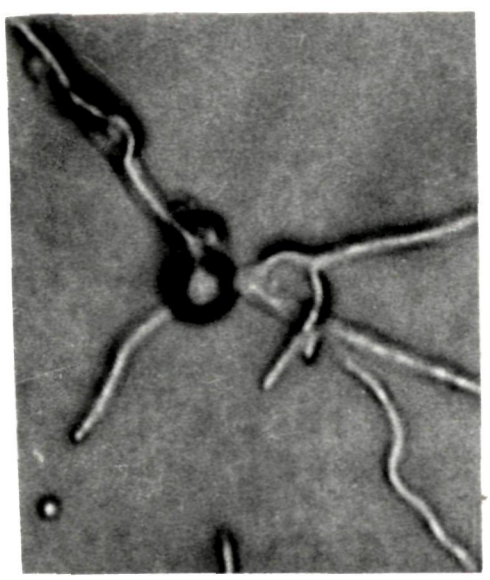


Fig. 5.5 c

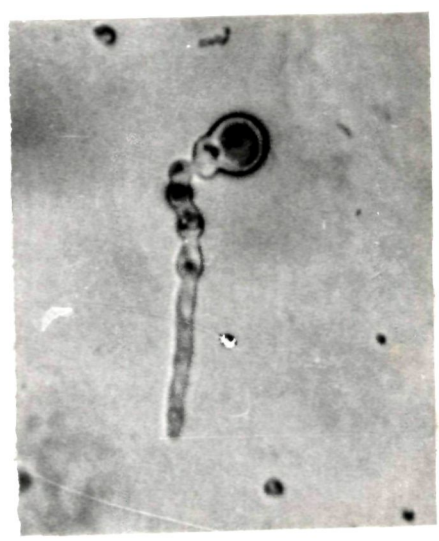


Fig. 5.5 d

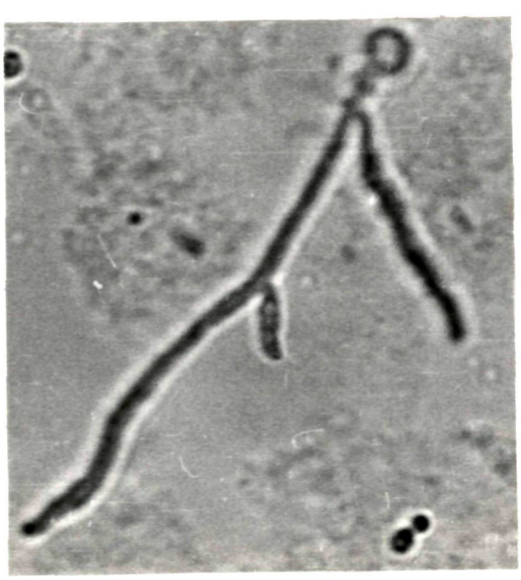


Fig. 5.5 e

Fig. 5.5 f

hyphal morphology after formation of a germination tube from one of the buds (Fig. 5.5 d). This kind of a regeneration behaviour has been termed as dimorphic regeneration in case of fungi (Necas and Svoboda, 1985).

In regeneration media (MYG containing 0.6 M sucrose) containing lower concentration of agar (less than 0.5 % w/v) a predominance of multiple germination tubes (2 - 4 per protoplast) was observed. The percentage of the budding-type forms remained unaffected by the percentage of agar used to solidify the regeneration medium (defined as the hardness of medium by Kobayashi *et al.*, 1985).

5.2.3 Studies on the secretion of starch- and pullulan-hydrolysing enzymes by free and immobilized (calcium alginate beads) protoplasts

5.2.3.1 Secretion of the amylolytic enzymes by free and immobilized protoplasts

Although the optimum yields of protoplasts was observed with 24 h old mycelium, protoplasts obtained from 48 h old mycelium were used for the studies on secretion of different amylolytic activities. This was due to the observation that extracellular amylolytic activities could only be detected in protoplasts obtained from 48 h old mycelium. The results of the enzyme secretion patterns of the free and immobilized protoplasts induced with soluble starch (1% w/v) are summariazed in Table 5.3.

5.2.3.2 Hydrolysis of starch by free and immobilized protoplasts of S. rolfsii

It was observed that both free as well as immobilized protoplasts of *S. rolfsii* were capable of hydrolysing tapioca and potato starches (1% w/v). The dynamics of production of glucose in the medium and the disappearence of starch (followed by the reduction in the iodine binding capacity) are shown in Fig. 5.6 and 5.7. The immobilized protoplasts were found to be capable of hydrolysing the tapioca and

Fig. 5.6: Production of glucose in the induction medium and the relative decrease in absorbance in the iodine-binding capacity of tapioca starch (1% w/v) at 600 nm. The values of decrease in absorbance of iodine binding at 600 nm are expressed as percent of the initial readings (0 h).

Glucose production ($mg.ml^{-1}$):

(▽——▽) Free protoplasts

(▲——▲) Immobilized protoplasts

Relative absorbance at 600 nm (%):

(⊙——⊙) Free protoplasts

(●——●) Immobilized protoplasts

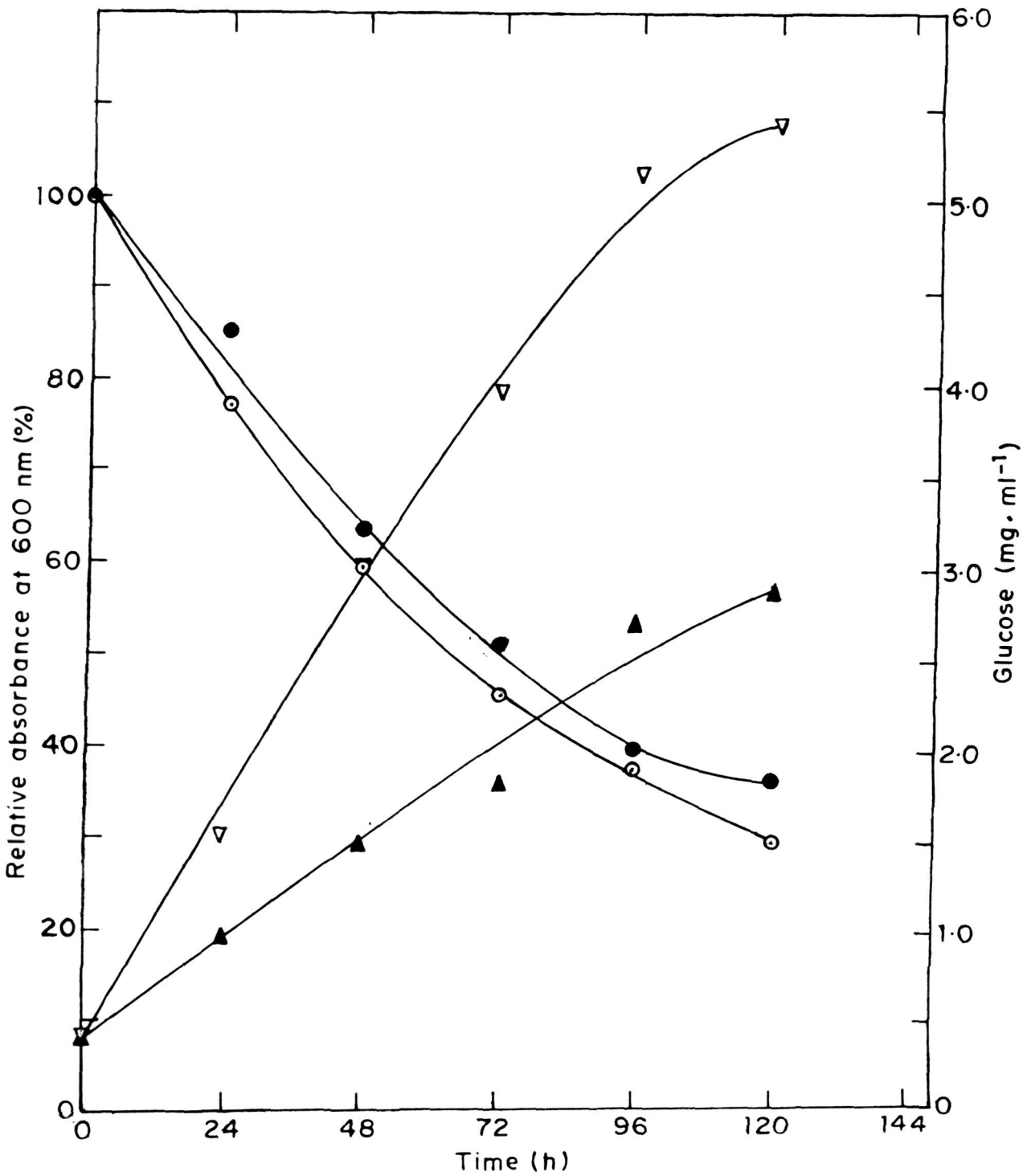

Relative absorbance at 600 nm (%)
Glucose (mg. ml⁻¹)
Time (h)
0
20
40
60
80
100
1·0
2·0
3·0
4·0
5·0
6·0
24
48
72
96
120
144

Fig. 5.7: Production of glucose in the induction medium and the relative decrease in absorbance in the iodine-binding capacity of potato starch (1% w/v) at 600 nm. The values of decrease in absorbance of iodine binding at 600 nm are expressed as percent of the initial readings (0 h).

Glucose production ($mg.ml^{-1}$):

(▽——▽) Free protoplasts

(▲——▲) Immobilized protoplasts

Relative absorbance at 600 nm (%):

(⊙——⊙) Free protoplasts

(●——●) Immobilized protoplasts

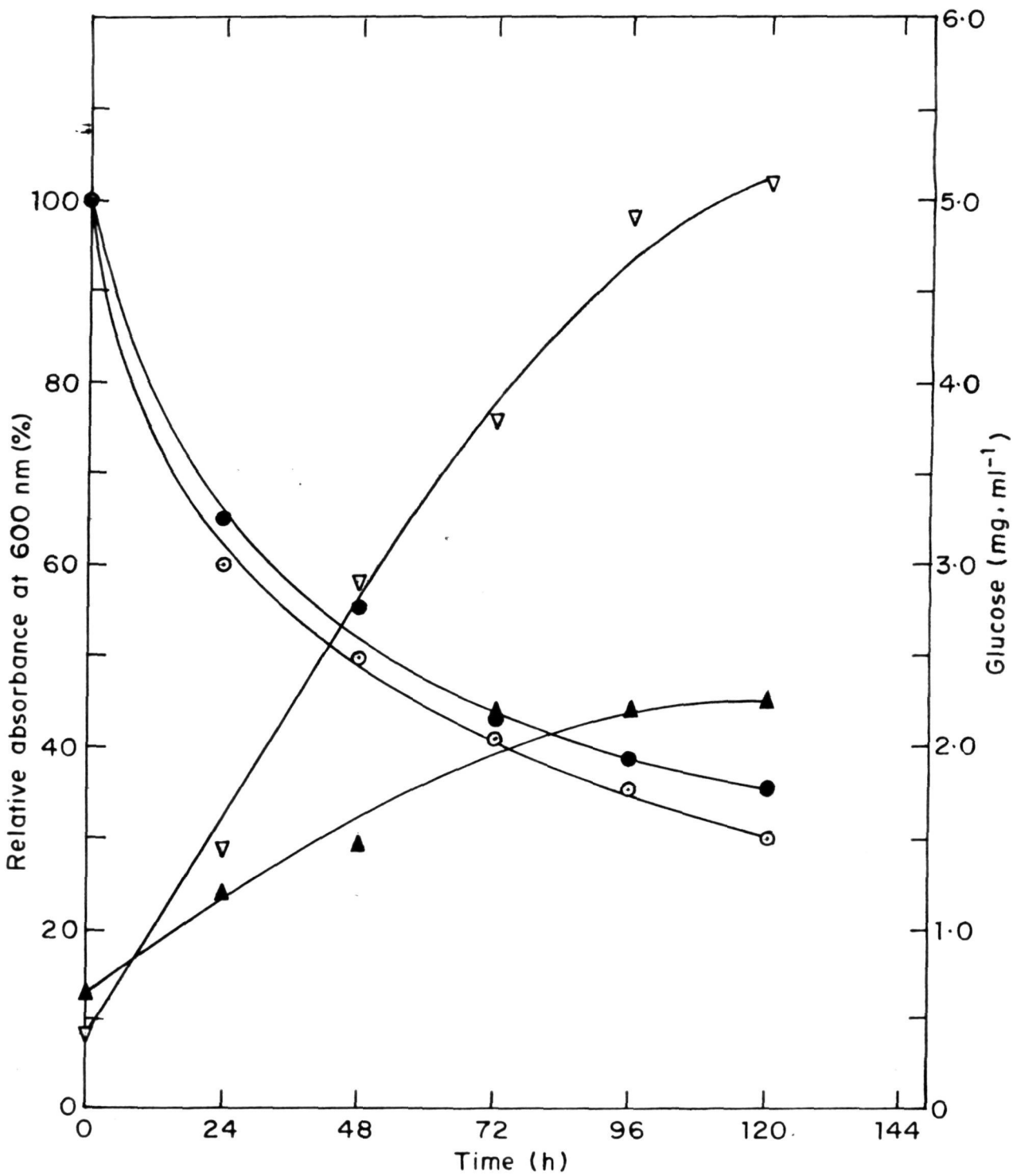

Relative absorbance at 600 nm (%)
Glucose (mg . ml⁻¹)
Time (h)
0
20
40
60
80
100
1·0
2·0
3·0
4·0
5·0
6·0
24
48
72
96
120
144

Table 5.3 : Secretion of various amylolytic activities by free and immobilized protoplasts of *S. rolfsii*[a]

Time[b]	α-Amylase		Glucoamylase		Pullulan-hydrolysing activity	
	IU X 10^{-2}. ml^{-1}					
(h)	F	I	F	I	F	I
0	-	-	-	-	-	-
24	1.8±0.5	1.0±0.1	4.1±0.7	2.0±0.2	1.1±0.4	N.D.
48	2.6±0.6	2.3±0.2	5.7±0.8	2.6±0.3	1.6±0.3	0.9±0.05
72	2.3±0.3	2.8±0.3	5.5±0.8	3.2±0.3	1.2±0.2	1.1±0.1
96	2.0±0.2	2.0±0.2	4.9±0.7	2.5±0.3	0.9±0.2	1.2±0.2
120	1.6±0.2	1.7±0.2	3.5±0.7	2.2±0.2	0.9±0.1	1.0±0.05

a Activities as described in section 2.3.6

b Time after immobilization.

Note : F, free protoplasts; I, immobilized protoplasts,

N.D. not detected.

potato starches with a conversion efficiency of 52 - 57% and 42 - 48%, respectively (in terms of the glucose produced at the end of 96 h), when compared with the free protoplasts.

5.2.3.3 Operational stabiliy of the immobilized protoplast preparation

The reusability of the immobilized protoplasts was adjudged based on their ability to produce the pullulan-hydrolysing activity. It was found that the immobilized protoplast preparation exhibited a half-life (57% of the initial activity retained) of the order of 6 d (three cycles of 48 h each). The viability of the protoplasts at the end of third cycle was found to be affected, (checked by dissolving the beads in phosphate buffer, pH 6.5, containing 0.6 M sucrose) as evidenced by the decrease in the regeneration frequency to less than 10%.

The results presented in this chapter have been published earlier (Kelkar *et al.*, 1990).

5.3 DISCUSSION

With the establishment of isolation of protoplasts from filamentous fungi by the use of commercially available enzyme preparations (Hamlyn *et al.*, 1981), there has been a fresh stimulus for the studies with fungal protoplasts. The various mycolytic preparations used for the isolation of protoplasts of filamentous fungi are complex mixtures of a number of enzyme activities. Featured prominantly in these are, β-(1->3)- and β-(1->6)-glucanases, α-(1->3)-glucanases, chitinases and proteases (Peberdy, 1985; Davis, 1985). The role of chitinases in effecting the release of protoplasts from fungi appears to be crucial because of the significant proportion of

chitin present in the fungal cell walls (Teng and Whistler, 1973). The initial objective of the present investigations was the standardization of a rapid, reproducible protocol for the isolation of protoplasts from *S. rolfsii*.

In *S. rolfsii* nearly 60% of the cell wall fraction is reported to be composed of chitin (Bloomfield and Alexander, 1967). The success of NovoZym 234 for effecting efficient release of protoplasts from *S. rolfsii* can thus be attributed to the significant levels of chitinase present in the enzyme preparation. On this background, the failure of Onozuka R-10 (which is predominantly a cellulase containing enzyme) to effect the rapid release of protoplasts, as observed by Deshpande *et al.* (1987), was not surprising.

Of the various concentrations of NovoZym 234 tested, a concentration of 5 $mg.ml^{-1}$ was found to be optimum for the release of protoplasts. Increasing the concentration of the enzyme further affected the isolated protoplasts adversely (Fig. 5.1). Similar observations have been made by Bej and Perlin (1989) while studying the effect of concentration of NovoZym 234 on the protoplasts isolated from *Ustilago violacea*.

A wide range of inorganic stabilizers have been used for the stabilization of free protoplasts liberated from filamentous fungi (Davis, 1985). The choice of the osmotic stabilizer is known to be strongly dependent on the conditions used for the isolation of protoplasts and on the organism under consideration. Protoplasts obtained in presence of KCl (0.6 M) and NaCl (0.7 M) were found to be stable in case of *S. rolfsii*. Bej and Perlin (1988) however, have found that the protoplasts of *U. violacea* had to be stabilized by KCl at a concentration of 1.2 M. Such differences observed in the concentration of the stabilizer required have been attributed to the differences in the normal internal osmotic pressure observed in the different organisms (Villaneuva

and Garcia-Acha, 1971).

In case of *S. rolfsii*, though protoplasts were efficiently released when $MgSO_4$ (0.6 M) and $MgCl_2$ (0.7 M) were used as osmotic stabilizers, they were found to be fragile. Contrasting results have been reported in case of *Lentinus edodes* (Kawasumi *et al.*, 1987); *Hebeloma cylindrosporum* (Hebraud and Fevre, 1988) and *Sclerotium glucanicum* (Deed and Seviour, 1989), where $MgSO_4$ (0.7 - 1.2 M) was observed to be the stabilizer of choice.

In case of filamentous fungi, sugars and sugar alcohols generally do not support efficient isolation of protoplasts (Davis, 1985). With *S. rolfsii* no protoplasts were isolated when sucrose (0.6 M) was tried as an osmotic stabilizer. Sorbitol (0.4 M) was also found to give very reduced yields of protoplasts. Recently, Deed *et al.* (1990) observed that with mannose and sucrose as osmotic stabilizers, no protoplasts were released from the mycelium of *Acremonium diospyri* ATCC 1066 using NovoZym 234. Billich *et al.* (1988) have also reported similar results with the protoplasts from *Fusarium scirpi*. However, on this background, good protoplast isolation has been reported using sugars and sugar alcohols from *Blastomyces dermatitidis* (0.9 M sorbitol, Crowe *et al.*, 1987); *Oudemansiella mucida* (0.6 M glucose, Homolka, 1988) and *Termitomyces clypeatus* (0.5 M sorbitol, Mukherjee and Sengupta, 1988). This again emphasizes the strong dependence of efficiency of isolation of protoplasts on the organism under consideration besides the type and the concentration of the stabilizer used.

Resistance of the older mycelium to the digestion by lytic enzymes has been reported in several cases (Picataggio *et al.*, 1983; Crowe *et al.*, 1987; Collings *et al.*, 1988; Homolka, 1988; Billich *et al.*, 1988). This build-up of resistance has been

attributed to the melanization of the cell walls in case of the older cultures of fungi (Bull, 1970 a,b). Melanin was thought to affect the action of various lytic enzymes by masking of the cell wall components as well as by direct inhibition. As suggested by Van den Broek *et al.* (1979), the changing composition of the fungal cell wall (in response to the age of the culture and the environmental conditions) may also affect the degree of digestion observed. Bartnicki-Garcia and Lippman (1972) had suggested that during the exponential growth phase, the high concentration of the cell wall building enzymes present in the mycelia may be complementing the action of the extracellular mycolytic enzymes added. This may effectively result in the enhancement of isolation of protoplasts from the mycelia in the exponential phase of growth. In case of *S. rolfsii*, as the culture ages it starts producing copious amount of an extracellular polysaccharide ("scleroglucan"), which may be further preventing an effective contact between the cell wall and the mycolytic enzymes. This is evidenced by a sharp drop in the efficiency of protoplast isolation in older cultures of *S. rolfsii* (Fig. 5.3).

In general, the release of protoplasts from filamentous fungi is documented to be initiated from the apical regions of the hyphae. These are observed to swell and protoplasts are released by the pinching-off of the cytoplasm that protrudes from the pores produced in the wall by the lytic enzymes (Homolka, 1988). In contrast to this observation, the initiation of protoplast release in case of *S. rolfsii* was found to be predominantly from the sub-apical regions of the hyphae. Similar exceptions have also been noted for *Neurospora crassa* (Quigley *et al.*, 1987); *S. glucanicum* (Deed and Seviour, 1989) and *A. diospyri* ATCC 1066 (Deed *et al.*, 1990).

Pretreatment of the mycelium with thiol compounds has been documented to promote the release of protoplasts from certain fungi (Davis, 1985). In some of the cases, the pretreatment, which is thought to destabilize the sulfide linkages present in the cell wall, is found to be absolutely essential e.g. *Acremonium chrysosporium* (Hamlyn *et al.*, 1981); *Condiobolus lampragues* (Ishikawa and Oishi, 1985) and *A. diospyri* ATCC 1066 (Deed *et al.*, 1990). In case of *S. rolfsii,* there was no benificial effect of the pretreatment of the myclium with thiol compounds, with respect to the number of protoplasts or the time required for the release of the protoplasts. This was in accordance with similar observations that have been made for *Beauvaria bassiana* (Pfeiffer and Khachatourians, 1987) and *S. glucanicum* (Deed and Seviour, 1989).

Regeneration frequencies of the protoplasts of *S. rolfsii* were found to be significantly improved with sucrose (0.6 M, regeneration frequency ~ 75%) as an osmotic stabilizer, instead of KCl (0.6 M, 2%). An exceptionally high regeneration frequency of 99.6% has been reported for the protoplasts of *A. niger* M-13 by Yabuki *et al.* (1984) using mannitol (1 M) as the osmotic stabilizer. Regeneration frequencies of the order of 60% were reported for the protoplasts of *Geotrichum candidum* by Jacobsen *et al.* (1985). Similar results have been reported in the case of *Volvariella volvacea* (Mukherjee and Sengupta, 1988) and *C. lampragues* (Ishikawa and Oishi, 1985). In general, the regeneration frequencies are found to be less than 30% in majority of the reports (Kropp and Fortin, 1985; Ijima and Yanagi, 1986; Anne-Brown *et al.*, 1986; Quigley *et al.*, 1987; Homolka, 1988; Hebraud and Fevre, 1988; Deed *et al.*, 1990). In presence of NH_4Cl as well as sorbitol, the protoplasts of *S. rolfsii* showed

In case of *S. rolfsii*, the resuspension of the protoplast pellet in MYG medium containing sucrose (0.6 M), initiated the synthesis of the cell wall. On solid regeneration media, the protoplasts were found to become insensitive to the osmotic shock after 5 - 6 h of incubation signifying the completion of the synthesis of the cell wall. In case of liquid regeneration media, this lag extended up to 12 h. Kobayashi *et al.* (1985) have attributed the longer lag that was observed in the case of protoplasts regenerating in the liquid media to the loss of some of the wall material which was being synthesized by the regenerating protoplasts by diffusion. Using the thin layer agar (TLA) method of Kobayashi *et al.* (1985), a microcolony from the regenerated protoplast became apparent under the microscope at the end of 24 h. Visible colonies from the regenerting protoplasts of *S. rolfsii* appeared after 3 - 4 days of incubation at 30°C on petri plates.

Regenerating protoplasts in case of filamentous fungi exhibit two types of growth. On completion of the cell wall synthesis the protoplast may give rise to a germ tube like structure, that then branches out and proliferates to give a normal mycelial morphology ("isodiametric regeneration"). On the other hand, in some cases, irregular budding-type forms may be seen in the regenerating protoplasts ("anisodiametric regeneration"). This phase of growth is observed to be a transient one and the budding structure later on forms a germ tube and reverts to normal mycelial morphology. This phenomenon has been termed as "dimorphic regeneration" (Necas and Svoboda, 1985). So far, the phenomenon has been reported only in few fungi e.g. *T. reesei* (Picataggio *et al.*, 1983); *Piricularia oryzae* (Kobayashi *et al.*, 1985); *T. clypeatus* (Mukherjee and Sengupta, 1988); *Sclerotium glucanicum* (Deed and Seviour, 1989) and *A. diospyri* (Deed *et al.*, 1990).

In case of *S. rolfsii*, a small fraction (0.1%) of the regenerating protoplasts was found to show anisodiametric regeneration. Kobayashi *et al.* (1985) tried to correlate the physical environment of the regeneration to the type of regeneration observed in culture. They reported that allowing the protoplasts of *P. oryzae* to regenerate in either liquid media or in media containing low concentrations of agar (less than 0.5% w/v) resulted in a predominance of the budding-type of forms in the regenerating protoplasts. An agar concentration of more than 0.5% (w/v) was found to support the regeneration of majority of the protoplasts by the formation of a germ tube (Kobayashi *et al.*, 1985). In case of *S. rolfsii*, however, the percentage of the budding forms was found to be unaffected by the concentration of the agar used in the medium. A predominance of multiple germination tubes (2-4 per protoplast) was observed when the agar concentration used in the medium was less than 0.5% (w/v). Recently, it was observed by Deed *et al.* (1990) that the dimorphic regeneration in the case of *A. diospyri*, could be controlled by the state of the regeneration medium. Liquid Czapek-Dox broth supplemented with NaCl (0.7 M) was found to favour the budding-type of forms, whereas on solid media, regeneration was observed to occur by the formation of germ-tubes only. Such a distinction could not be seen in case of *S. rolfsii*.

The use of protoplasts as a cell wall less experimental system for the production of metabolites as well as macromolecules is a field that has received less attention. The identification of *T. reesei* protoplasts as viable physiological units for the secretion of carboxymethyl cellulase and β-glucosidase activities by Kolar *et al.* (1985) provided the first impetus for the studies of secretion of enzymes by the protoplasts. These studies were prompted by the observation that, the secretion of the extracellular enzymes in fungi was hampered, as the enzymes remain bound to or

within the cell wall for some time (Kubicek, 1981). Following these studies, the protoplasts of *S. rolfsii* were shown to be capable of secretion of carboxymethyl cellulase and β-glucosidase by Deshpande *et al.* (1987).

The amylolytic enzyme activities were detectable in the protoplasts of *S. rolfsii* (derived from 48 h old mycelium) induced by soluble starch. Hence, their potential for the secretion of different amylolytic activities was evaluated both in the free as well as the immobilized form. Comparison of the secretion patterns of the amylolyic activities by the protoplasts of *S. rolfsii* showed that the maximum enzyme levels were obtained in the case of free protoplasts after 48 h of incubation, whereas for the immobilized protoplasts required 72 h. The increase in the time required for attaining the maximum levels of the enzyme activities in the immobilized protoplasts can be attributed to the additional diffusion barriers present in this case. The protoplasts in the presence of an inorganic stabilizer were found to be adversely affected. The decrease in the enzyme levels observed after 96 h of incubation, was correlated to continued presence of the inorganic stabilizer which supports very low frequency of regeneration. Sucrose, could not be used to replace KCl as the osmotic stabilizer, since in presence of sucrose, both the free and the immobilized protoplasts reverted rapidly to mycelial forms.

Since, the protoplasts were observed to secrete amylolytic activities extracellularly, their potential for production of glucose from commercial starches was evaluated. Compared to the free protoplasts, the immobilized preparation was able to hydrolyze the tapioca and the potato starches (1% w/v) with a conversion efficiency of 55% and 44%, respectively (in terms of glucose produced at the end of 96 h). The degradation of the starch was also evident from the fall in the iodine binding capacity of the starch present.

The preparation of the immobilized protoplasts exhibited a half-life of 6 days (three cycles of 48 h each), when secretion of the pullulan-hydrolysing activity was used as a marker (57% activity remaining). The decrease in the activity was due to the loss of viability of the protoplasts as evident from the drop in regeneration frequency to less than 10%.

A suitable procedure for rapid isolation and high frequency regeneration of *S. rolfsii* protoplasts - the essential prerequisite for the protoplast mutagenesis and fusion procedures, was developed. Additionally, the potential application of immobilized protoplasts of *S. rolfsii* for hydrolysis of starch was explored.

CONCLUSION

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Pullulan, an exopolysaccharide from *Aureobasidium pullulans*, is a homopolymer of glucose composed of repeating maltotriose units that are polymerized on the terminal glucose residues by the α-(1->6)-glycosidic linkages. Pullulan has been used as a model substrate for starch-debranching enzymes. Microorganisms capable of pullulan-hydrolysis, therefore become potential sources of starch-debranching enzymes.

Pullulanase (EC 3.2.1.41) endo-hydrolyses pullulan resulting in the accumulation of maltotriose in the hydrolysates. The most extensively investigated pullulanase is derived from *Klebsiella oxytoca* (formerly *Aerobacter aerogenes*, *K. pneumoniae*). Apart from pullulanase, there are four major groups of enzymes that have been reported to hydrolyse pullulan. These are isopullulanase (EC 3.2.1.57), neopullulanase and amylopullulanase (both tentative designations). Apart from these, glucoamylase (EC 3.2.1.3) has also been reported to act on pullulan in a few reports. In general, there are few reports of enzymes from fungal sources capable of hydrolysis of pullulan in literature.

In the initial part of the work, the production of the enzyme activity was optimized by the manipulation of different medium constituents provided for growth. Hydrolysed starch (1 - 3% w/v) was found to be the best carbon source supporting optimum production of the extracellular pullulan-hydrolysing activity (1.05 - 1.62 $IU.ml^{-1}$). Supplementation of different organic additives such as corn steep liqour in the medium was found to be favorable for the production of pullulan-hydrolysing activity.

These studies were followed by some investigations on the thermostability of the pullulan-hydrolysing enzyme activity in presence of some polyhydric alcohols. It was determined that in absence of any additive, the pullulan-hydrolysing activity in the culture supernatant had a half-life of the order of 30 min at 60°C, a temperature normally used for various starch hydrolytic processes. Sorbitol (3.0 M) was observed to completely protect the enzyme activity from inactivation, with practically no loss of enzyme activity even after 7 h of incubation at 60°C.

The purification and the characterization of the enzyme activity was therefore undertaken subsequently. The purification of the enzyme was achieved using conventional chromatographic procedures such as ion-exchange chromatography (DEAE-Cellulose DE-52) and gel permeation chromatography (Bio-Gel P-150). The purified enzyme showed a single band in disc gel electrophoreses carried out at pH 2.9 and 7.5. The enzyme showed a single band on SDS-polyacrylamide gel electrophoresis on treatment with SDS (1% w/v) and 2-mercaptoethanol (1% v/v) with a molecular mass of about 64,000 daltons. This corresponded with the native protein indicating that the enzyme was most probably composed of a single polypeptide chain. It was observed that during the purification procedures the pullulan-hydrolysing activity was always accompanied by the starch-hydrolysing activity. The specific activities (μmol of glucose equivalents.min^{-1}.mg^{-1} protein) of the enzyme for the hydrolysis of pullulan and soluble starch were found to be 3.0 and 89.7, respectively. The enzyme was found to hydrolyse starch as well as pullulan optimally in the pH range of 4.0 - 4.5 (0.05 M acetate buffer) and was stable in a similar pH range of (3.5 - 5.0) with retention of atleast 75% of the initial enzyme activity after 15 min at 50°C.

The temperature optimum for the hydrolysis of pullulan was determined to be 50°C, whereas the hydrolysis of starch was observed to proceed optimally at 65°C. This difference in the optimum temperature for hydrolysis of the two substrates can be attributed to the differences in the chemical compositions of the substrates. The predominance of the non-reducing end groups in starch may provide a stabilizing effect for the enzyme activity. A similar observation was made for the pullulan-hydrolysing glucoamylase "S" from *Cladosporium resinae* by McCleary and Anderson (1980). From the Arrhenius plots, the activation energy for the hydrolysis of pullulan was found to be 19.9 $kJ.mol^{-1}$, where as for the hydrolysis of starch it was determined to be 25.9 $kJ.mol^{-1}$.

The K_m values for the hydrolysis of pullulan and soluble starch were determined to be 5.13 and 0.6 $mg.ml^{-1}$, respectively, at pH 4.9 (0.05 M acetate buffer) and 50°C.

No significant inhibition of the starch-, as well as the pullulan-hydrolysing activities of the enzyme was noted by most of the metal ions tested (e.g. Ca^{++}, Co^{++}, Mn^{++}, Mo^{++}, Li^{+} and Mn^{++} at 0.005 M each). On the other hand, Cu^{++}, Hg^{++}, Pb^{++}, Fe^{+++} and EDTA were found to be inhibitory.

The enzyme was found to hydrolyse various substrates containing α-(1->4)- and α-(1->6)-glucosidic linkages e.g. pullulan, starch, amylose, amylopectin, glycogen and dextran. The product of hydrolysis of all these substrates was noted to be glucose. Depending on these observations, the enzyme was classified as a 1,4-α-D-glucan glucohydrolase (glucoamylase, EC 3.2.1.3). The enzyme was capable of hydrolysis of α-(1->6)-glycosidic linkages, as was evident from the hydrolysis of dextran. Predominantly β-linked substrates such as cellulose, chitin and

xylan were not hydrolysed by the enzyme. Inhibition studies with acarbose (an analog of maltotetraose) ruled out the presence of a contaminating α-(1->6)-endo-hydrolysing enzyme activity. It also suggested that the that the hydrolysis of all the substrates may be taking place at the same catalytic site.

Studies on the secretion of extra-cellular enzymes in case of fungi are hampered by the fact that the enzymes remain bound to or within the cell wall for some time. Fungal protoplasts offer an attractive experimental tool to overcome some of these difficulties. Investigations on the ability of protoplasts of *S. rolfsii* to secrete amylolytic enzymes were undertaken. Optimization of the parameters was carried out to standardize a protoplast system for *S. rolfsii* which was rapid, reproducible and released viable protoplasts in adequate numbers. In a typical protocol, it was standardized that, 50 mg of the *S. rolfsii* mycelium (24 h old) incubated with NovoZym 234 (5 mg) in one ml of 0.05 M maleic acid-NaOH buffer (pH 5.0) gave the maximum yields of protoplasts (5 - 6 X 10^6) in 4 h. A significant regeneration frequency (~ 70%) was achieved for the protoplasts of *S. rolfsii* using sucrose (0.6 M) as the osmotic stabilizer.

Protoplasts of *S. rolfsii*, free as well as immobilized in calcium alginate beads, were found to secrete amylolytic enzymes when incubated with soluble starch (1% w/v) in osmotically stabilized acetate buffer (pH 5.0, 0.6 M KCl). The immobilized protoplasts in addition were able to hydrolyse tapioca and potato starches (1% w/v) with conversion efficiencies of 27 and 22 %, in terms of the glucose produced at the end of 96 h. The entrapped protoplast were determined to have a functional half-life of 6 d when incubated at 30°C (three cycles of 48 h each), when production of the pullulan-hydrolysing activity was used as a marker.

In conclusion, a pullulan-hydrolysing enzyme (glucoamylase) was isolated and characterized from the culture filtrate of *S. rolfsii*. The pullulan-hydrolysing ability of this glucoamylase distinguishes it from the other reported fungal glucoamylases, which do not hydrolyse pullulan efficiently. This observation, can prove to be of practical significance for the hydrolysis of starch by using amylolytic enzymes from *S. rolfsii*.

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LIST OF PUBLICATIONS

Research papers:

1. Hemant S. Kelkar, Anil H. Lachke and Mukund V. Deshpande

Extracellular constitutive production of pullulan-hydrolysing activity by *Sclerotium rolfsii*.

Can. J. Microbiol., **34**, 82-85 (1988)

2. Hemant S. Kelkar, V. Shankar and Mukund V. Deshpande

Rapid isolation and regeneration of *Sclerotium rolfsii* protoplasts and their potential application for starch hydrolysis.

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3. Hemant S. Kelkar and Mukund V. Deshpande

Effect of additives on the thermostability of amylolytic activities from *Sclerotium rolfsii*.

Communicated to *Enzyme and Microbial Technology*

Papers at Symposia and Meetings:

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Studies on the rapid isolation of *Sclerotium rolfsii* protoplasts and secretion of starch-hydrolysing activity.

- Hemant S. Kelkar and Mukund V. Deshpande

Pullulanase activity from *Sclerotium rolfsii*.

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