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Production of β-galactosidase from thermophilic fungus *Rhizomucor* sp

SA Shaikh, JM Khire and MI Khan

Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

A thermostable β-galactosidase was produced extracellularly by a thermophilic *Rhizomucor* sp, with maximum enzyme activity (0.21 U mg^{-1}) after 4 days under submerged fermentation condition (SmF). Solid state fermentation (SSF) resulted in a nine-fold increase in enzyme activity (2.04 U mg^{-1}). The temperature range for production of the enzyme was 38–55°C with maximum activity at 45°C. The optimum pH and temperature for the partially purified enzyme was 4.5 and 60°C, respectively. The enzyme retained its original activity on incubation at 60°C up to 1 h. Divalent cations like Co^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} had strong inhibitory effects on the enzyme activity. The K_m and V_{max} for *p*-nitrophenyl-β-D-galactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside were 0.39 mM, 0.785 mM and 232.1 mmol min^{-1} mg^{-1} respectively. The K_m and V_{max} for the natural substrate lactose were 66.66 μM and 0.20 μmol min^{-1} mg^{-1}.

Keywords: β-galactosidase; thermophilic fungus; *Rhizomucor*; extracellular; solid state fermentation

Introduction

The enzyme β-galactosidase (β-d-galactohydrolase EC 3.2.1.23) catalyses the hydrolysis of lactose to glucose and galactose. It is a commercially important enzyme and its use in the dairy and food industry is well known. Its occurrence in nature is diverse ie in plants, animals, organs and microorganisms [6]. The commercially available β-galactosidases have been mainly obtained from yeast and fungi, the most important sources being *Kluyveromyces lactis*, *Kluyveromyces fragilis* [3] and *Aspergillus niger* [6]. Enzymes derived from thermophilic microorganisms which show a higher degree of thermostability are preferred because of their various advantages, such as they are less prone to microbial contamination, the higher temperature reduces the viscosity of the reaction mixture and higher rates of reaction can be achieved [20]. Although thermostable β-galactosidase from mesophilic organisms like *Alternaria alternate* [10], *Neurospora crassa* [4], *Sclerotina sclerotiorum* [5] and thermophilic organisms like *Bacillus stearothermophilus* [7], *Thermus aquaticus* [22] etc have been reported, there are very few reports on extracellular production of the enzyme.

Most of the β-galactosidases, even those available commercially are intracellular in nature making their use uneconomical. Yeast are still the most important source of this enzyme. *Aspergillus oryzae* is the only fungal source of extracellular β-galactosidase that has been used commercially [18]. The enzyme has been generally obtained through submerged fermentation.

In recent years, solid state fermentation (SSF) has acquired importance because of economical and practical advantages [11,13,17,19]. Besides these, another important advantage of solid state fermentation is that most of the secondary metabolites which are produced in extremely low yields in submerged fermentation can be produced on a comparatively large scale by this method [8]. Moreover, the conditions in which the fungus grows in solid state are similar to its natural growth conditions [9]. A system for producing β-galactosidase from yeast by solid state fermentation has been reported [1]. However there is no report on extracellular β-galactosidase production either from mesophilic or thermophilic fungus by solid state fermentation.

The production and partial purification of extracellular β-galactosidase from thermophilic fungus *Rhizomucor* sp by solid state fermentation and a comparison of the enzyme obtained by submerged and solid state fermentation is reported in the present communication.

Correspondence: Dr MI Khan, Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India
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Materials and methods

Culture

The thermophilic fungus *Rhizomucor* sp was isolated from soil samples and maintained on potato dextrose agar (PDA) slants containing (per liter of distilled water): extract from 200 g potatotes; glucose, 20 g; yeast extract, 1.0 g; and agar, 20 g. The *Rhizomucor* sp is deposited with the National Collection of Industrial Microorganisms (NCIM), Division of Biochemical Sciences, National Chemical Laboratory, Pune, India, with accession No. 1253.

Medium and culture conditions

The basal medium for submerged fermentation contained (per liter of distilled water): yeast extract, 10 g; lactose, 10 g; K_2HPO_4, 1.0 g and $MgSO_4 \cdot 7H_2O$, 0.5 g. The medium (50 ml) was distributed in 250-ml Erlenmeyer flasks and each flask was inoculated with 1.0 ml spore suspension containing 10^6 spores per ml from a 7-day-old sporulated culture grown on PDA slant at 45°C. The flasks were incubated on a rotary shaker at 45°C at 200 rpm. The mycelium was removed from the culture broth by filtering through Whatman No. 1 under vacuum and the clear filtrate was used for determining β-galactosidase activity.

Solid state fermentation was carried out in 250-ml Erlenmeyer flasks containing wheat bran, 9.5 g; lactose, 0.5 g; K_2HPO_4, 0.1 g; and $MgSO_4 \cdot 7H_2O$, 0.05 g and was moistened with 20 ml distilled water. Each flask was inoculated with 1.0 ml of spore suspension containing 10^6 spores from a 7-day-old sporulated culture grown on PDA slant at 45°C. The flasks were incubated at 45°C. The enzyme was extracted by suspending the solid in 50.0 ml of 20 mM citrate-phosphate buffer, pH 4.5 containing 0.85% NaCl. The suspension was kept shaken at 150 rpm for 90 min. The mycelium was separated from the fermentation medium by filtering through muslin cloth followed by Whatman No. 1. The clear filtrate was used to determine β-galactosidase activity.

Estimation of biomass

The biomass was hydrolyzed according to the method of Nishio [16] and the glucosamine was estimated by the method of Blix [2].

Scanning Electron Microscopy (SEM)

Samples for SEM were fixed with ultra-violet light and were mounted on brass stubs. Specimens were then coated with a thin layer of gold (100 Å) in a gold coating unit, model E 5000, Polaron Equipment Ltd, UK, and were viewed with an SEM Leica Stereoscan 440, UK, at an accelerating voltage of 10 kV, and beam current 25 Pa. The photographs were recorded by 35 mm camera attached to the high-resolution recording unit.

Partial purification of β-galactosidase

The culture filtrate obtained from submerged fermentation and the enzyme extract from solid state fermentation were precipitated with ammonium sulfate at 90% saturation and the precipitate was dialyzed against 20 mM phosphate buffer, pH 7.2. The dialysate was then applied on DEAE-cellulose column. The adsorbed enzyme was eluted batchwise with 20 mM phosphate buffer, pH 7.2 containing NaCl (0.1–0.5 M) and was dialyzed against the same buffer. The dialysate was used to study the enzyme properties.

Enzyme assay

β-Galactosidase was assayed by incubating 25 μl of suitably diluted enzyme with 50 μl of 6.6 mM *p*-nitrophenyl-β-d-galactopyranoside (pNPG) or *o*-nitrophenyl-β-d-galactopyranoside (ONPG) and 925 μl of 20 mM citrate-phosphate buffer, pH 4.5 at 60°C for 30 min. The reaction was terminated by adding 1.0 ml of 0.5 M Na_2CO_3 and the *p*-nitrophenol or *o*-nitrophenol released was determined by reading the absorbance at 405/420 nm respectively. When lactose was used as substrate, the enzyme activity was assayed using a glucose oxidase-peroxidase kit to determine the amount of glucose liberated [21]. One unit of β-galactosidase activity (U) was expressed as the amount of enzyme that releases 1.0 μmol of product (*p*-nitrophenol/*o*-nitrophenol/d-glucose) per minute under standard assay conditions. Protein was measured by the method of Lowry [14] with bovine serum albumin as standard.

All experiments were carried out in triplicate and the values reported are the mean of three such experiments in which 3–5% variability was observed.

Electrophoresis

Native gel electrophoresis of the ammonium sulfate precipitated and DEAE-cellulose adsorbed and eluted enzyme was carried out according to the method of Laemmli (7.5%, pH 8.0) [12] and the protein bands were visualized by staining with silver nitrate. β-Galactosidase activity was also checked by cutting the gel into equal pieces (~1.0 cm). Each piece was crushed and the protein was extracted in citrate phosphate buffer, 20 mM, pH 4.5, and assayed for β-galactosidase activity.

Molecular weight

The partially purified enzyme was loaded on a Sephacryl S-300 gel filtration column to determine the approximate molecular weight of the partially purified protein. The column was calibrated using standard molecular weight markers: bovine serum albumin (66 kDa); alcohol dehydrogenase (150 kDa); apoferratin (443 kDa); and thyroglobulin (669 kDa).

Results

Isolation of the fungus

A variety of different fungi were isolated from soil samples collected randomly. Single spore isolates were inoculated on YpSs agar plates and incubated at 45°C. These isolates were screened for β-galactosidase activity in liquid medium containing yeast extract and lactose. One of the isolates, *Rhizomucor* sp deposited with NCIM, with accession No. 1253 producing extracellular β-galactosidase was selected for further work.

Effect of carbon sources on production of β-galactosidase

The effect of various carbon sources (1%) indicates that although all sugars supported the growth of the fungus, only wheat bran and lactose induced β-galactosidase activity. Increase in β-galactosidase activity was observed with an increase in lactose concentration. Maximum activity was obtained in the present of 1% lactose in SmF and 5% lactose in SSF. A further increase in the concentration of lactose did not enhance the enzyme activity (Table 1). An equivalent amount of enzyme activity was observed when untreated milk whey was used as carbon source and moistening agent (Table 2). Among the various nitrogen sources tested (0.5%), yeast extract and peptone were superior to other sources for β-galactosidase production under submerged conditions, although all others supported the growth of the fungus (Table 3).

Time course of β-galactosidase production

The time course of β-galactosidase production in submerged and solid state conditions is shown in Figure 1. Under submerged conditions maximum enzyme activity, 0.55 U ml^{-1} (0.21 U mg^{-1}) was obtained on the 4th day of fermentation, while under solid state conditions, maximum enzyme activity 5.5 U ml^{-1} (2.04 U mg^{-1}) was obtained on the 5th day of fermentation. It was observed that the enzyme production was associated with biomass production.

Table 1 Effect of carbon sources on production of β-galactosidase activity in submerged fermentation

Carbon source (1%)	Final pH	Enzyme activity (U ml^{-1})	Wet wt of mycelium	Dry wt of mycelium
Glucose	8.10	0.003	2.20	0.32
Sucrose	8.10	0.002	1.24	0.19
Maltose	8.10	0.004	1.17	0.17
Xylose	8.10	0.003	1.23	0.19
Galactose	8.10	0.014	3.60	0.35
Wheat bran	8.10	0.034	0.61	0.10
Fructose	8.10	0.003	1.05	0.17
Mannose	8.10	0.003	2.20	0.31
Lactose				
0.5%	8.40	0.053	2.31	0.31
1.0%	8.40	0.550	3.58	0.38
2.0%	8.40	0.093	3.95	0.51
5.0%	8.40	0.053	6.07	1.40

The fungus was grown in a medium containing yeast extract and lactose was replaced by other sources.

Table 2 Effect of carbon sources on production of β-galactosidase by solid state fermentation

C-source	Final pH	Activity (U ml^{-1})	GlcNAc (mg g^{-1} dry mass)
Lactose			
1%	8.40	1.2	1.14
2%	8.40	3.2	2.08
5%	8.40	5.0	1.97
10%	8.40	2.1	2.67
Milk whey			
initial pH 5.0	8.40	4.8	2.18
initial pH 7.0	8.40	5.0	2.30

When lactose was used as a carbon source the wheat bran was moistened with 20 ml water. No water was added when whey was used.

Effect of initial pH on enzyme production

The effect of initial pH (3–10) of the medium on β-galactosidase production in submerged fermentation is shown in Table 4. Although the fungus grew over a wide pH range of 4–10, the enzyme was produced in a medium of initial pH between 7–8. Maximum enzyme activity was detected in a medium with initial pH 7.0.

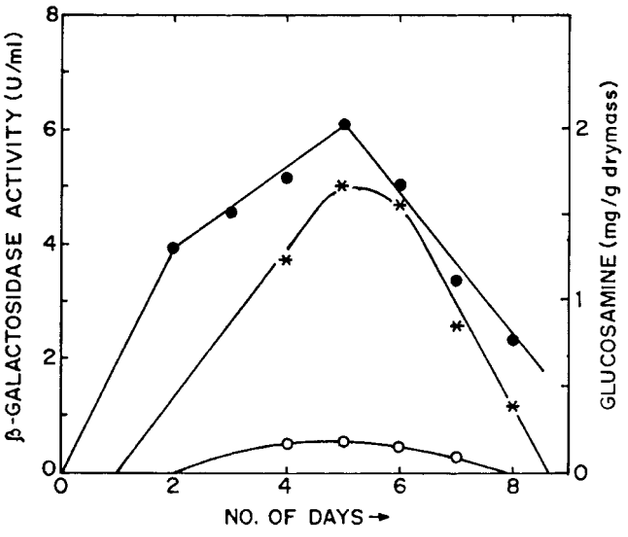

Figure 1 Time course of β-galactosidase production by *Rhizomucor* sp in submerged (—○—), solid state fermentation (—∗—) and biomass (glucosamine, mg g^{-1} dry mass) during solid state fermentation (—●—).

Table 3 Effect of nitrogen sources on production of β-galactosidase in submerged fermentation

Nitrogen source	Final pH	Enzyme (U ml^{-1})	Wet wt of mycelium (mg)
Yeast extract	8.3	0.55	2.21
Tryptone	7.1	0.05	1.31
Malt extract	6.0	0.03	0.53
Casamino acids	5.1	0.04	1.62
Peptone	8.1	0.50	2.74
Liver extract	7.1	0.05	1.43
Wheat bran			
1%	8.4	0.05	1.10
5%	8.4	0.15	1.76
7%	8.4	0.12	1.38
10%	8.4	0.08	0.96

The fungus was grown in a medium containing 1% lactose and 0.5% yeast extract. The yeast extract was replaced by the above nitrogen sources at a concentration of 0.5% (w/v).

Table 4 Effect of initial pH on enzyme production in submerged fermentation

Initial pH	Relative activity (%)
3.0	0.0
4.0	24.0
5.0	30.0
6.0	38.0
7.0	100.0
8.0	75.0
9.0	51.0
10.0	42.0

Desired pH of the medium was obtained by using HCl and NaOH.

Effect of metal ions on enzyme production

The effect of various metal ions (2 mM) on production of β-galactosidase was studied using various divalent metal ions (Table 5). The growth of the fungus was totally inhibited in the presence of Co^{2+}. Although Mn^{2+} and Zn^{2+} supported the growth of the fungus, the enzyme activity was inhibited considerably. Similarly Ca^{2+}, Cu^{2+} and Fe^{2+} were also inhibitory towards the production of the enzyme.

Effect of temperature on production of β-galactosidase

The effect of different temperatures (37–55°C) on production of β-galactosidase is shown in Figure 2. The enzyme secretion was maximum at 45°C in the case of SmF and SSF. At 60°C the fungus did not grow.

Partial purification of β-galactosidase

The crude enzyme obtained in SmF showed specific activity of 0.21 U mg^{-1}. The maximum amount of the DEAE cellulose adsorbed enzyme was eluted with 0.2 M NaCl in 20 mM phosphate buffer pH 7.2 (specific activity, 10.83 U mg^{-1}) showing 48-fold purification. A similar increase in specific activity was seen in case of the enzyme obtained from solid state fermentation (Table 6). This increase in specific activity was reflected in Figure 3 where most of the protein impurities were eliminated after the DEAE step. The protein band corresponding to β-galactosidase was identified by assaying the gel pieces as described in materials and methods (Figure 3, lane 2).

Table 5 Effect of metal ions on enzyme production in submerged fermentation

Metal ion	Relative activity
None	89.0
Mg^{2+}	100.0
Ca^{2+}	62.0
Cu^{2+}	60.0
Zn^{2+}	14.0
Fe^{2+}	35.0
Mn^{2+}	10.0
Co^{2+}	0.0

The fungus was grown with the above salts (2 mM) in the fermentation medium at 45°C in submerged conditions as described in materials and methods. Mg^{2+} was replaced by other salts.

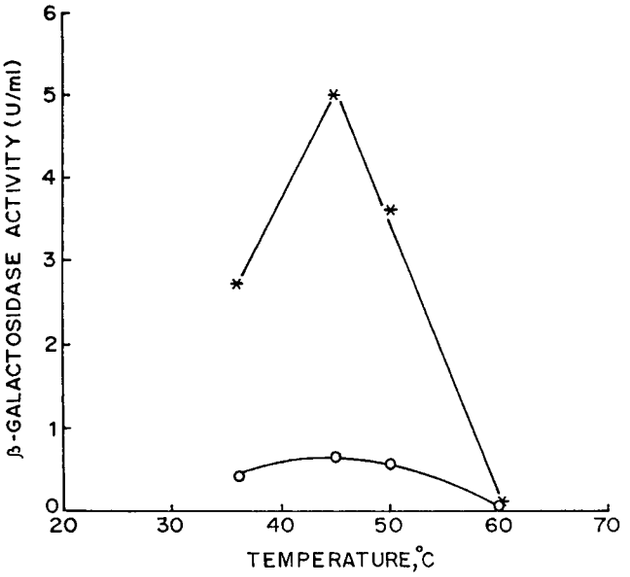

Figure 2 Effect of temperature on production of β-galactosidase in solid state (—∗—) and submerged fermentation (—○—).

Effect of pH on enzyme activity and stability

The effect of pH on the β-galactosidase activity produced by SmF and SSF was studied by incubating the enzyme in buffer of pH range 3–8 (Figure 4). The optimum pH was found to be 4.5 and the enzyme was stable over a wide pH range ie 3–8 at 4°C for 24 h. At pH 9.0, the enzyme retained 70% of its original activity.

Effect of temperature on enzyme activity and stability

The effect of temperature on enzyme activity and stability is shown in Figures 5 and 6 respectively. The optimum temperature for the enzyme activity was 60°C. The enzyme was stable at this temperature for 50 min. After 120 min 67% of the original activity was retained. At 70°C the enzyme retained 73% and 20% of its original activity after 10 min and 60 min respectively. Similar results were obtained in the case of SSF.

Effect of metal ions on enzyme activity

The effect of metal ions on the enzyme activity is shown in Table 7. Ni^{2+} has no effect on enzyme activity. The enzyme was strongly inhibited by Hg^{2+} but to a smaller extent by other metal ions tested. Co^{2+} showed some enhancement in enzyme activity.

The K_m and V_{max} for pNPG and ONPG were 0.39 mM, 0.785 mM and 232.1 mmol min^{-1} mg^{-1}, 38.67 mmol min^{-1} mg^{-1} respectively. The K_m and V_{max} for the natural substrate lactose was 66.66 μM and 0.20 μM min^{-1} mg^{-1} (Table 8).

The approximate molecular weight of the partially purified enzyme was found to be 250 kDa by gel filtration.

SEM studies

The structure of uninoculated wheat bran is shown in Figure 7a. The growth of *Rhizomucor* was observed after 72 h (Figure 7b). Dense mycelial growth on the surface of solid substrate is seen in Figure 7c. The penetration of fungal hyphae into the substrate particles and its ability to

Table 6 Purification of β-galactosidase from *Rhizomucor* sp

	Submerged fermentation		Solid state fermentation	
	(U mg^{-1})	(fold purification)	(U mg^{-1})	(fold purification)
Culture filtrate	0.21	1	2.04	1
Ammonium sulphate precipitate	1.19	5.6	3.07	1.5
DEAE-cellulose	10.18	48.4	21.42	10.07

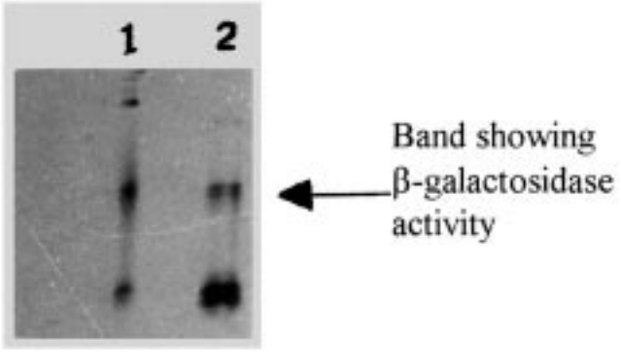

Figure 3 Native gel electrophoresis of partially purified β-galactosidase. Lane 1: ammonium sulfate fractionated (90% saturation) enzyme and lane 2: DEAE cellulose-purified protein.

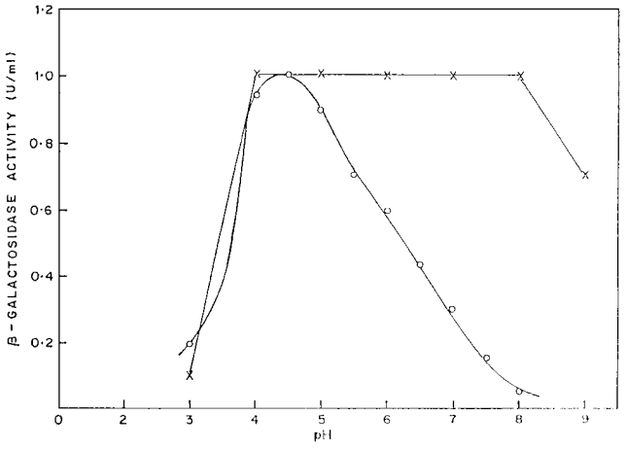

Figure 4 Effect of pH on β-galactosidase activity (—○—) and stability (—X—). For optimum pH, β-galactosidase activity was measured using desired buffers (20 mM citrate-phosphate and sodium phosphate) at 60°C for 30 min. For pH stability, the enzyme was kept at 4°C for 24 h and then used for activity measurements.

soften the substrate by mechanically pushing the cells apart in conjunction with enzymatic degradation has been reported [15].

Discussion

The results obtained in the present work show an increase in yield (2.04 U mg^{-1}) of β-galactosidase by the *Rhizomucor* sp in solid state fermentation as compared to submerged fermentation (0.21 U mg^{-1}). The enhanced activity obtained in solid state fermentation is a result of increased

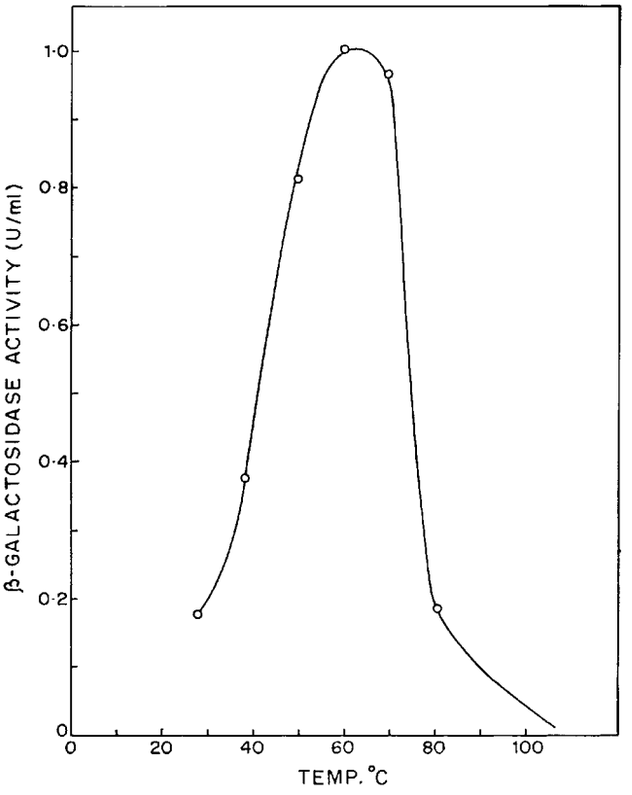

Figure 5 Effect of temperature on enzyme activity. The enzyme was incubated in 20 mM citrate-phosphate buffer, pH 4.5, at various temperatures for 30 min.

biomass indicated by the N-acetylglucosamine content as seen in Figure 1. The enzyme production is found to be lactose-dependent. An increase in lactose concentration shows an increase in β-galactosidase activity and maximum activity is obtained at 1% lactose concentration in submerged fermentation and 5% lactose in case of solid state fermentation. Similar enhancement in the production of the enzyme by *Kluyveromyces lactis* in solid state fermentation [1], and by *Aspergillus oryzae* in semisolid fermentation [16] has been reported. The enzyme shows preference for pNPG as compared to ONPG, however the best affinity is shown towards lactose, the natural substrate of the enzyme. Increase in yield using wheat bran may prove useful in economizing the use of fungal β-galactosidase for industrial applications which are otherwise uneconomical. The combination of high temperature stability and low pH optima helps eliminate microbial spoilage during processing.

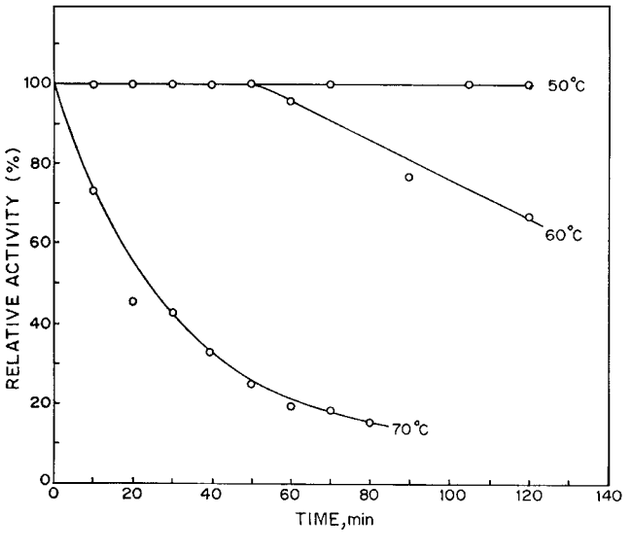

Figure 6 Effect of temperature stability of enzyme. The enzyme was incubated at the temperatures indicated for various intervals of time, then cooled in ice and the residual activity was measured.

Table 7 Effect of metal ions on enzyme activity

Metal ion (2 mM)	Relative activity (%)
Control	100.0
Zn^{2+}	75.0
Ni^{2+}	95.0
Hg^{2+}	0.0
Fe^{2+}	74.0
Ca^{2+}	85.0
Co^{2+}	133.0
Mn^{2+}	61.0
Mg^{2+}	63.0
Cu^{2+}	74.4
EDTA	97.0

β-Galactosidase was obtained by the method described in materials and methods. The metal ions to be tested were added to the assay system and the results expressed as a percentage of the control.

Table 8 Substrate specificity for β-galactosidase

Substrate	K_m (mM)	V_{max} (mmol mg^{-1} min^{-1})
pNPG	0.388	232.1
ONPG	0.785	38.67
Lactose	0.066	0.002

Further work on the purification and characterization of the enzyme is in progress.

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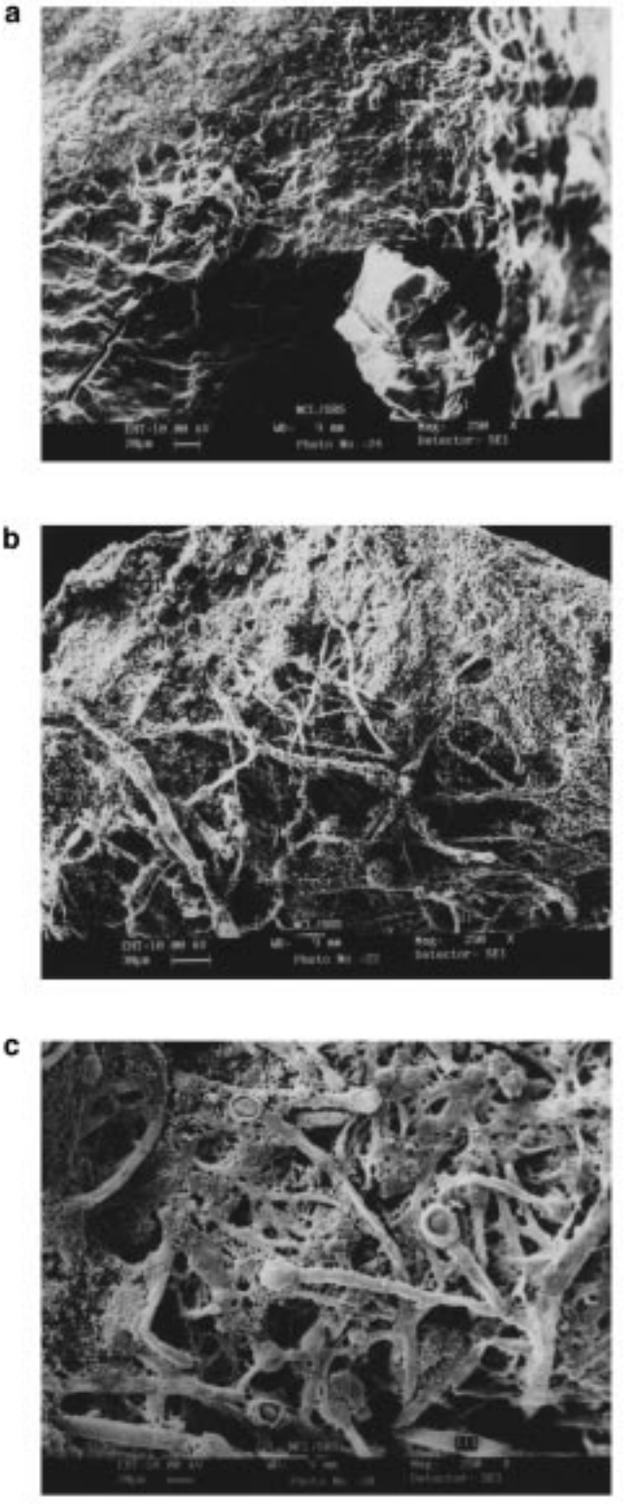

Figure 7 Scanning electron microscopy. (a) Uninoculated wheat bran; (b) Growth of *Rhizomucor* after 72 h; (c) Growth after 6 days.

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