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Purification and characterization of maltooligosaccharide-forming amylase from *Bacillus circulans* GRS 313

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A maltooligosaccharide-forming amylase that hydrolyzes starch into maltotriose and maltopentaose was found in the culture filtrate of a strain of *Bacillus circulans* GRS 313 isolated from local soil. The enzyme was purified by organic solvent fractionation, Sephadex G-100 gel filtration and CM-Sephadex column chromatography. Optimum pH and temperature of amylase were evaluated using response surface methodology (RSM) and were found to be 48°C and 4.9, respectively. The enzyme was stable up to 60°C and its pH stability was in the range of 5.0–8.0. The K_m and V_{max} of the amylase with starch were 11.66 mg/ml and 68.97 U, respectively, and the energy of activation, E_a, was 7.52 kcal/mol. Dextrin inhibited the enzyme competitively, with a K_i of 6.1 mg/ml, and glucose caused noncompetitive inhibition with a K_i of 9.5 mg/ml. The enzyme was inhibited by Hg^{2+}, Mn^{2+}, Fe^{3+} and Cu^{2+} and enhanced by Co^{2+} and Mg^{2+}. EDTA reversed the inhibitory effect of the metals. Paper chromatographic and high-performance liquid chromatography analysis of the products of the amylolytic reaction showed the presence of maltotriose, maltotetraose, maltopentaose, maltose and glucose in the starch hydrolysate.
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Introduction

Staling of baked goods is generally defined as an increase in crumb firmness and corresponding loss in product freshness. The staling is generally accepted to be caused by a gradual transition of starch from an amorphous structure to a partially crystalline state. The increase in starch crystallinity by an intermolecular or intramolecular association of starch molecules *via* hydrogen bonds is known as retrogradation [12]. The maltooligosaccharide-forming amylases reduce retrogradation and thus find application in the baking industry as antistaling agents [9,22].

The literature on production of maltooligosaccharide-forming amylase from *Bacillus circulans* is sparse [29]. Moreover, the isolated amylase from *B. circulans* MG-4 yields mainly maltotetraose from liquefied starch with small amounts of glucose, maltose, maltotriose and other higher maltooligosaccharides, but not maltopentaose. A constitutive extracellular maltotetraose-producing amylase was isolated from *Pseudomonas* sp. IMD 353 [11]. Chung *et al* [6] reported an amylase from *Thermococcus profundus* DT 5432, which hydrolyzed starch to produce α-maltose and maltotriose and smaller amounts of higher maltooligosaccharides. More recently, two high maltose-forming α-amylases from *Penicillium expansum* and *Aspergillus oryzae* were studied by Doyle *et al* [8]. An amylase that produced maltotretraose and maltopentaose was found in the culture filtrate of *B. circulans* GRS 313, which was isolated from the local soil. The present work was undertaken with the purpose of characterizing the enzyme. The effect of pH and temperature on the purified amylase was studied using a statistical experimental design method (response surface methodology, RSM), which aimed at optimizing the conditions for the amylolytic reaction. Knowing the optimal conditions for the enzymatic reaction, the kinetic and thermodynamic constants and the other catalytic properties of the enzyme were evaluated.

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Materials and methods

Materials

CM-Sephadex and Sephadex G-100 were products of Pharmacia Biotech (Sweden). Soluble starch was supplied by E Merck (Germany) and 1,3 dinitrosalicylic acid (DNS) was purchased from Lancaster (England). Bovine serum albumin (BSA) was a Sigma (USA) product.

Microorganism and its characteristics

The organism was isolated from the local soil and was characterized according to *Bergey's Manual of Systematic Bacteriology* [27]. The properties of the isolate were:

Property	*B. circulans* GRS 313
Gram character	Gram-positive
Shape	rods, motile
Spore	oval, endospores in subterminal position
Growth under aerobic condition	+
Nutrient agar colony	circular, opaque, irregular
Growth temperature	22–50°C

pH for growth	5.0–11.0
Growth in NaCl	2.5–6.5% NaCl
Growth under anaerobic condition	+
Indole test	+
Methyl red test	+
Voges Proskauer test	−
Citrate utilization	−
Casein hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	+
Nitrate and nitrite reduction	+
Catalase test	+
Gelatin liquefaction	+
Utilization of sugars	arabinose, cellobiose, dextrose, fructose, maltose, xylose, salicin, melibiose, raffinose, rhamnose.

B. circulans GRS 313 was cultivated in 100 ml of medium containing soybean meal 48.4 g/l, yeast extract 15.8 g/l and wheat bran 28.4 g/l [7]. The culture broth was centrifuged for 20 min at 10,000 rpm and the supernatant was used for enzyme purification.

Purification of enzyme

All purification operations were performed at 4°C. The culture filtrate was concentrated using cold acetone in the ratio of 1:2. The concentrate was dialyzed against 0.01 M acetate buffer, pH 4.0. The concentrated enzyme was loaded onto a Sephadex G-100 column (2.6×64 cm) equilibrated with the same buffer. The active fractions were pooled and concentrated by lyophilization. The lyophilized enzyme solution was loaded onto a CM-Sephadex column (2.6×14.5 cm) equilibrated with 0.1 M acetate buffer (pH 4.0). After the column was washed with the same buffer, the enzyme was eluted with 0.1 M phosphate buffer (pH 8.0). The pooled fractions were dialyzed against 0.05 M acetate buffer (pH 6.0) and used as the purified enzyme.

Polyacrylamide gel electrophoresis (PAGE) and protein analysis

Analysis of the samples during protein purification was performed using sodium dodecyl sulfate (SDS) PAGE [19]. Protein content in the chromatographic fraction was estimated by measuring the absorbance at 280 nm. The protein content in the enzyme fraction was determined by the method of Lowry *et al* [20] using BSA as standard.

Amylase assay

Amylase activity was assayed with dinitrosalicyclic acid according to a modified Bernfeld method [2]. The activity was measured in a 1-ml reaction mixture containing 0.5 ml of 2% (wt/vol) starch solution in 0.01 M acetate buffer, pH 4.5, and enzyme solution. The reaction was incubated at 50°C for 10 min. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar under the standard conditions.

Statistical analysis

RSM with two factors, pH and temperature, at five levels each, was used to study the simultaneous effects of these reaction parameters on the amylase activity and to evaluate the optimum pH and temperature for the enzymatic reaction. The amylase activity was regarded as the response value and 12 experimental runs were performed according to a 2^2 factorial Central Composite Experimental Design (CCD) [4,5,16]. The range of variables was fixed according to the preliminary trials, for pH from 4.0 to 4.9 and for temperature from 43°C to 57°C. The variable levels X_i were coded as x_i according to the following equation, such that X_0 corresponded to the central value:

$$x_i = (X_i - X_0)/\Delta X_i, \qquad i = 1, 2, 3, \ldots, k$$

where x_i was the dimensionless value of an independent variable; X_i was the real value of an independent variable; X_0 was the real value of an independent variable at the center point, ΔX_i was the step change.

The results were analyzed and expressed as a second-order model:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where Y_i was the activity of the enzyme, which was regarded as the predicted response; $x_i x_j$ were input variables, which influenced the response variable Y; β_0 was the offset term; β_i was the ith linear coefficient; β_{ii} was the ith quadratic coefficient; and β_{ij} was ijth interaction coefficient.

The value of the dependent response was the mean of two replications. The second-order polynomial coefficients were calculated using SYSTAT (8.0; HSS Inc., USA). The analysis of difference between means was evaluated by ANOVA and F-test using SYSTAT (8.0; HSS), Finally, the results were expressed as a contour plot using MATLAB (version 4; The Math Works, MA, USA).

Temperature and pH stability of the enzyme

The thermal stability of the enzyme was examined after preincubation of the enzyme at 50, 60, 70 or 80°C for 120 min and carrying out the enzyme assay as described earlier. The effect of pH on the stability of the amylase was evaluated by incubating the enzyme in buffer solution of different pH values (0.01 M acetate buffer for pH 4.0–5.0; 0.01 M phosphate buffer for pH 5.5–7.5; 0.01 M Tris–glycine buffer for pH 8.0–9.0) for 1 h at 30°C after which the enzyme activity was determined.

Determination of kinetic constants

The initial reaction rate was determined at different substrate concentrations ranging from 1% (wt/vol) to 5% (wt/vol) (pH 4.9) and after incubation at 48°C and 57°C; the amount of enzyme solution was 0.5 ml containing 0.06 mg of protein. The incubation time of the reaction mixture was 10 min. The enzyme assay was performed as described earlier. The kinetic constants were evaluated using Lineweaver–Burk plots.

Relative activity towards various substrates

The enzyme reaction in the presence of various substrates was performed using 0.5 ml of substrate solution (2% wt/vol) prepared in 0.01 M acetate buffer of pH 4.9 and 0.5 ml of the enzyme

Table 1 Purification scheme for maltooligosaccharide-forming amylase from *B. circulans* GRS 313

Treatment step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	% Yield
Culture filtrate	100	98.3	15.6	6.3	100
Acetone precipitation	10	554.21	11.92	46.5	56.4
Gel filtration	16	82.48	0.99	83.2	13.4
CM-Cellulose	10.5	23.73	0.12	197.8	2.54

solution. The relative activity towards various substrates was calculated as:

$$\% \text{ Relative activity} = \text{Activity on substrate} \times 100/\text{Activity on soluble starch}$$

Inhibition studies

The inhibition studies were performed in the presence of high concentrations of glucose (50, 75 and 100 mM) and dextrin (4% wt/vol, 6% wt/vol, 8% wt/vol). The reaction mixture contained 0.5 ml of soluble starch, 0.5 ml of enzyme solution and 1 ml of inhibitor solution. The inhibition constant, K_i, was calculated according to the method outlined by Todhunter [31].

Analysis of hydrolysis product by paper chromatography and high-performance liquid chromatography (HPLC)

A volume of 0.9 ml of starch (2%, wt/vol) with 0.3 ml of enzyme solution was incubated at 48°C for 2, 5, 10 and 15 min. The reaction was stopped by rapid cooling with cold HCl (1 M). The hydrolysate (100 μl) was applied on Whatman filter paper and the chromato-

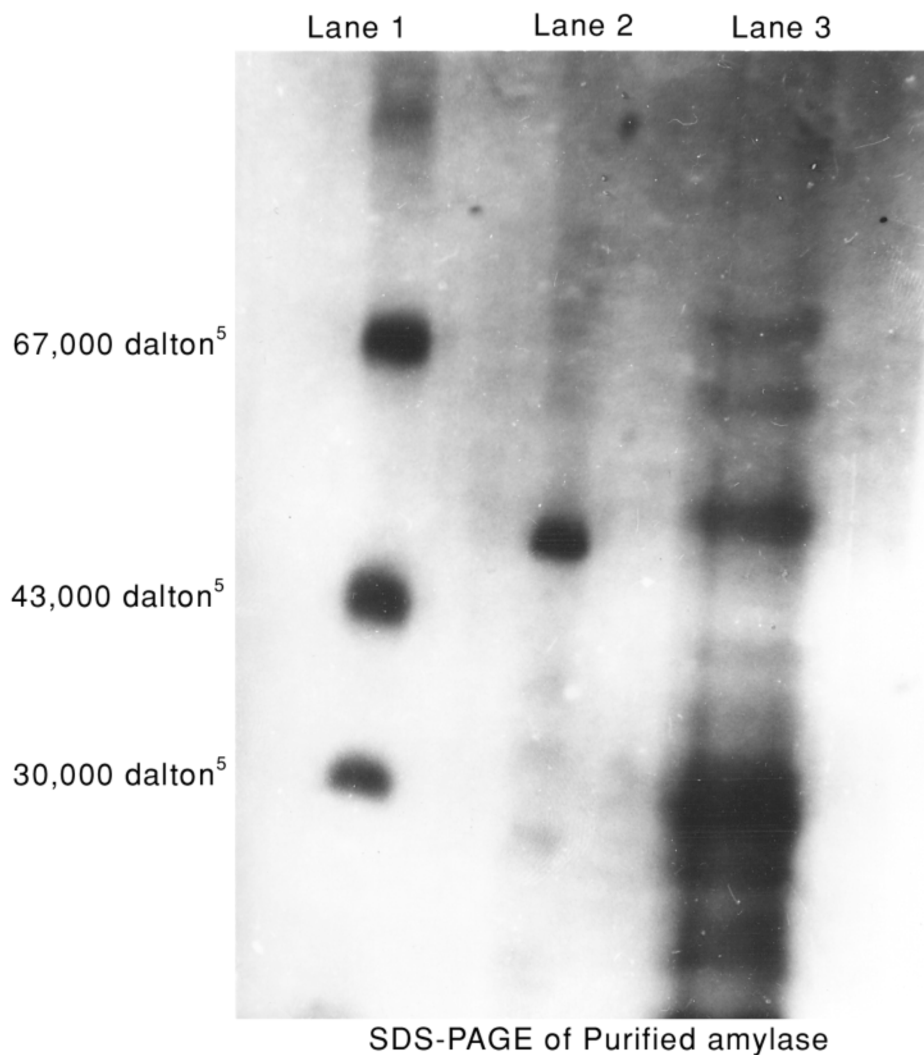

Figure 1 SDS-PAGE of purified amylase was performed using 10–20% gel. Lane 1: Molecular weight markers. Lane 2: CM-Cellulose fraction of amylase. Lane 3: Crude amylase. The standard proteins used as molecular weight markers were albumin (M_r 67,000), ovalbumin (M_r 43,000) and carbonic anhydrase (M_r 30,000) (Sigma-Aldrich, USA).

Table 2 Two-factored CCD for studying the effects of pH and temperature on the activity of the amylase

Experimental level			Coded level	
Run	X_1	X_2	x_1	x_2
1	55	4.2	1	−1
2	55	4.8	1	1
3	45	4.2	−1	−1
4	45	4.8	−1	1
5	50	4.5	0	0
6	50	4.5	0	0
7	50	4.9	0	$\sqrt{2}$
8	50	4.0	0	$-\sqrt{2}$
9	57	4.5	$\sqrt{2}$	0
10	43	4.5	$-\sqrt{2}$	0
11	50	4.5	0	0
12	50	4.5	0	0

gram was developed by a descending technique with the solvent system (water:butanol:acetic acid, 5:4:3) at room temperature for 16 h. The reducing sugars were detected on the chromatogram by the silver nitrate test [21]. The markers used were glucose, maltose, maltotetraose and maltopentaose (Sigma).

For HPLC analysis, the sample was centrifuged (13,000×*g*, 10 min at room temperature) and the supernatant was analyzed after filtration (0.2 μm pore size prep disc membrane filter; Bio-Rad, USA). The endproduct analysis was carried out on a Aminex HPX (87 P 300×7.8 mm) Bio-Rad column with distilled water at a flow rate of 0.6 ml/min. The retention times were compared with those of known standards of maltose, maltotriose, maltotetraose and maltopentaose (Sigma) with a reflective index detector (Perkin Elmer, USA) LC-30 RI detector.

Results and discussion

Purification of amylase

The purification procedure and results are summarized in Table 1. The ion exchange chromatography step in the purification protocol removed about 98% of the protein found in the crude amylase solution and increased the specific activity of the purified fraction by 197.8 U/mg. At the final stage, the enzyme was purified 31.4-fold and the recovered activity was 2.54%. The amylase from *B. circulans* MG 4 was purified 26-fold with a yield of 0.3% [29].

Molecular weight of the enzyme

The molecular weight of the amylase from *B. circulans* GRS 313, as estimated from SDS-PAGE (Figure 1), was around 48 kDa. The molecular weight of this amylase was higher compared to that of the amylase from *B. circulans* MG 4 (10 kDa). Molecular weights of α-amylase from some other *Bacillus* species have been reported: *B. acidocaldarius* (68 kDa), *B. amyloliquefaciens* (49 kDa) and *B. subtilis* (24–100 kDa) [10]. The constitutive maltotetraose-producing amylase from *Pseudomonas* sp. had a relative molecular mass of 63 kDa [11].

Table 3 ANOVA

Source	*df*	Sum of squares	Mean squares	*F* value	*P*>*F*
Blocks	1	0.0096	0.0096		
Model	5	352.48	70.496	325.767	0.000
Error	5	1.082	0.2164		
Total	11	353.5758	32.143		

R=coefficient of correlation=0.984; R^2=coefficient of determination=0.968.

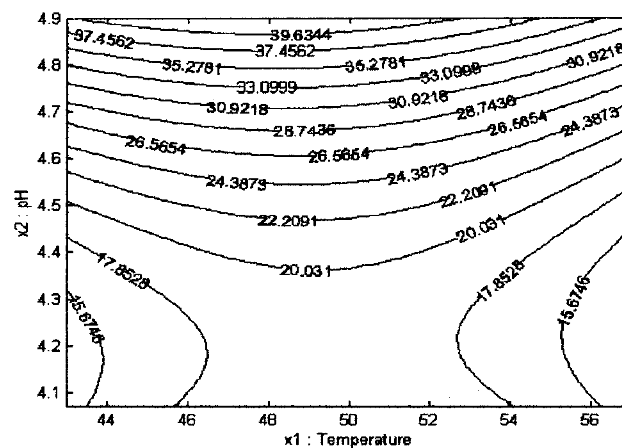

Figure 2 Contour plot: effect of temperature and pH on activity of the amylase.

Optimization of reaction pH and temperature using RSM

A summary of the CCD for optimization of the reaction pH and temperature is given in Table 2. The results were analyzed by a multiple regression procedure and the following model, relating the amylase activity with the independent process variables, temperature (X_1) and pH (X_2), was fitted:

$$Y = 510.816 + 11.2017X_1 - 366.7626X_2 - 0.0949X_1^2 + 46.2083X_2^2 - 0.4267X_1X_2$$

The goodness of fit of the regression equation was expressed as the multiple correlation coefficient, R, and the determination coefficient, R^2. The coefficient of determination, R^2, was 0.973, indicating that 97.3% of the sample variation could be explained by the model. The value of R for amylase activity was 0.986, showing a good agreement between experimental and predicted values. The corresponding analysis of variance (ANOVA) is summarized in Table 3.

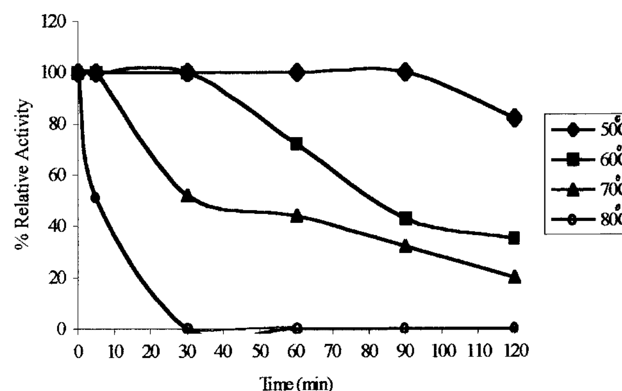

Figure 3 Thermostability of the amylase.

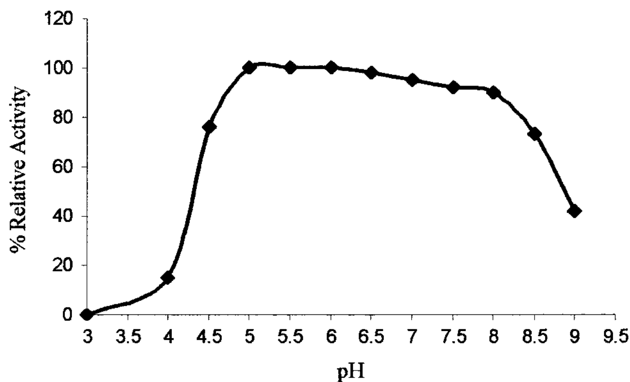

Figure 4 pH stability of the amylase. The enzyme was incubated at various pH for 1 h.

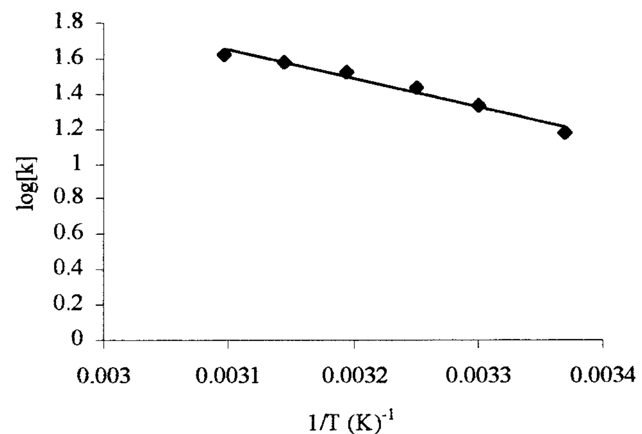

Figure 6 Arrhenius plot for determination of E_a.

The optimum pH and temperature, as evaluated from the contour plot (Figure 2), were 4.9 an 48°C, respectively. An increase in the amylase activity from 22.6 to 40.2 U/ml was observed after optimizing the process parameters using RSM. The maltooligosaccharide-forming amylase from *B. circulans* GRS 313 was active in the acidic range unlike other reported maltooligosaccharide-forming amylases. For instance, the optimum pH and temperature of the amylase from *B. circulans* MG-4 were 7.5 and 50°C, respectively [29]. A maltotriose-forming amylase from *Microbacterium imperiale* had a similar optimum temperature and pH of 50°C and 6.5–7.0, respectively [30]. The maltotetraose-forming amylase from *Pseudomonas stutzeri* and *P. saccharophila* exhibited optimum activity at pH 8.0 and 6.7, respectively [25].

Effects of temperature and pH on stability of the enzyme

The half-lives of the enzyme at 60°C and 70°C were 80 and 30 min, respectively, and at 80°C activity reached half within 5 min (Figure 3). The enzyme was more thermostable than the amylase from *B. circulans* MG-4, which was reported to be unstable above 40°C [29]. Maltopentaose-forming amylase from *Pseudomonas* sp. was stable up to 45°C [17] and maltotetraose-forming amylase from *P. stutzeri* rapidly lost activity above 40°C [25] as was the case with the maltotriose-forming amylase from *M. imperiale* [30]. An amylase from *Bacillus* sp. MD 124 was stable at 65°C for 60 min [15]. The amylase from *B. circulans* GRS 313 was stable between pH 5.0 and 8.0 (Figure 4) as reported for most of the *Bacillus* amylases [10]. However, there is a report of an amylase from *Bacillus* sp. MD 124 that was stable between pH 5 and 10.

Determination of kinetic constants, K_m and V_{max}

A comparison of the kinetic constants at two different temperatures, 48°C and 57°C, showed that the K_m of the enzyme increased with the increase in temperature from 11.66 to 19.11 mg/ml (Figure 5). The maximum reaction rate, V_{max}, decreased from 68.97 to 59.52 U with increasing temperature. This could be due to the fact that temperature affects the three-dimensional proteins structure of the enzyme molecule and there is a possibility that it also affects the substrate binding property of the enzyme, in turn affecting the K_m. The Michealis–Menten constant, K_m, was higher than the reported values for amylases from *B. subtilis* (0.63 mg/ml), *A. oryzae* EI 212 (3.86 mg/ml, at pH 5.0 at 50°C), *Lipomyces kononenkoae* (2.7 mg/ml, at pH 5.5 and 40°C) and *B. flavothermus* (2.2 mg/ml) [1,3,18,28].

Activation energy and energy change of enthalpy and entropy of the enzyme

The Arrhenius plot gave a single activation energy, E_a, for the enzyme at 7.54 kcal/mol (Figure 6). Studies on the activation energies of such maltooligosaccharide-forming amylases showed the existence of two distinct energies of activation, e.g., the maltotetraose-producing amylase from *P. stutzeri*, was reported

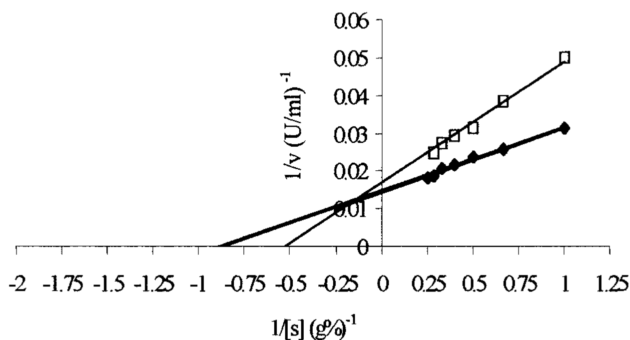

Figure 5 Determination of K_m and V_{max} by Lineweaver–Burk plot.

Table 4 Substrate specificity of the amylase

Substrate (2%, wt/vol)	% Relative activity
α-Cyclodextrin	0
Amylopectin	95
β-Cyclodextrin	0
Corn starch	94
Dextrin	96
Potato starch	100
Pullulan	43
Rice starch	90
Soluble starch	100
Wheat starch	89

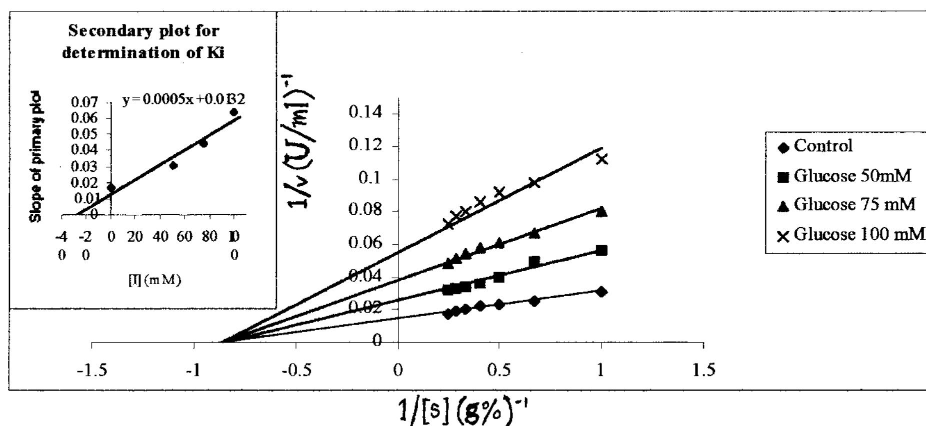

Figure 7 Noncompetitive inhibition of the amylase by glucose.

to have two E_a values, 13.4 and 5.2 kcal/mol [26]. Similar trends of dual E_a values were reported in the case of amylases from *B. subtilis* var. *amyloliquefaciens* and *P. saccharophila* [25]. The activation and deactivation energies of the partially purified enzyme from a newly isolated *Mucor* sp. were 11.13 and 37.5 kcal/mol [23]. The enthalpy change of activation (ΔH, kcal/mol) was calculated from the relationship. $\Delta H = E_a - RT$, where R is the gas constant (1.987 cal/mol). The free energy change (ΔG, kcal/mol) was then calculated from the Eyring equation as $\Delta G = RT \ln(k_B T/hk)$, where k_B is the Boltzmann constant (1.381×10^{-23} J/K) and h is the Planck constant (6.626×10^{-34} J s), k is the first-order reaction rate in s^{-1} and T is the absolute temperature [13]. The change in entropy (ΔS, cal/mol) was calculated from the thermodynamic relationship [14] $\Delta S = (\Delta H - \Delta G)/T$.

The thermodynamic constants for the amylase from *B. circulans* GRS 313 evaluated at 48°C and pH 4.9 were $\Delta H = 6.86$ kcal/mol, $\Delta G = 23.94$ kcal/mol, $\Delta S = -53.2$ cal/mol. The mechanism of action of amylase suggested by Robyt [24] proposes a reaction of the reactive intermediate with water stereospecifically before the product is released from the active site. Consideration of the proposed mechanism of amylase suggests that the negative entropy resulted from the restriction in the freedom of motion of the activated complex due to the bonding of the water molecules in the transition state. The enthalpy of the reaction was positive, indicating that the enzymatic hydrolysis was an endothermic reaction.

Relative activity towards various substrates

As shown in Table 4, soluble starch, wheat starch, corn starch, rice starch, potato starch, amylopectin, pullulan and dextrin were rapidly hydrolyzed by the enzyme but there was no effect on α- and β-cyclodextrin. The substrate specificity of the maltooligosaccharide-forming amylase *B. circulans* GRS 313 was slightly different from the amylase of *B. circulans* MG-4. The latter hydrolyzed β-cyclodextrin [29]. However, the present amylase was similar to the maltopentaose-forming enzyme from *Pseudomonas* sp., which did not have any effect on α- and β-cyclodextrin [17].

Inhibition studies

Glucose inhibited the enzyme noncompetitively with a K_i of 9.5 mg/ml. Figure 7 illustrates the effect of different concentrations of glucose on the enzyme hydrolysis and the secondary plot for determination of K_i. Dextrin was a competitive inhibitor of the enzyme with a K_i of 6.1 mg/ml (Figure 8). This is in accordance with the inhibition studies performed earlier [26] on a maltotetraose-forming amylase from *P. stutzeri*, which demonstrated competitive inhibition of the enzyme with dextran and maltotetraose.

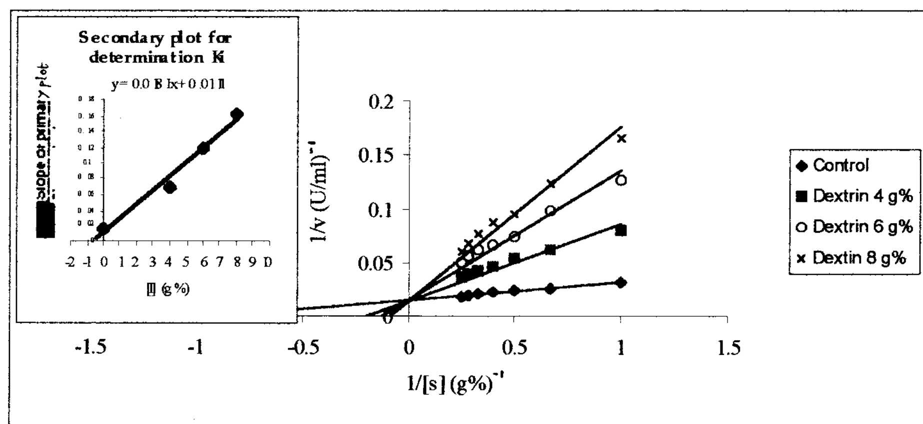

Figure 8 Competitive inhibition of the amylase by dextrin.

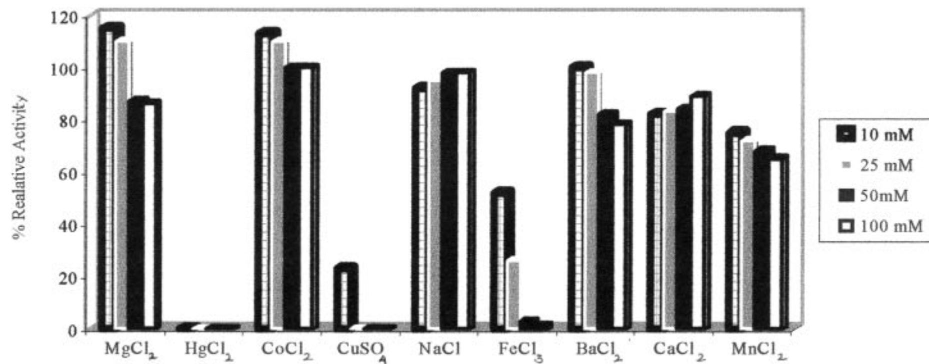

Figure 9 Effect of the metal ions on *B. circulans* GRS 313 amylase.

Effect of metal ions

Figure 9 shows the effects of various metal ions on the amylase activity. Co^{2+} and Mg^{2+} enhanced the enzyme action to a small extent at 10 and 25 mM concentrations. At 10 mM, Cu^{2+} and Fe^{3+} inhibited the activity by 23% and 52%, respectively. There was complete inhibition of enzyme action in the presence of Hg^{2+}. The inactivation of the enzyme by Cu^{2+} and Hg^{2+} indicates the presence of thiol groups or carboxyl groups in the enzyme molecule. These results corroborated the conclusions of earlier studies on the catalytic groups of amylases which implied that carboxylate anions act as nucleophiles [24]. The inhibitory effect of (10 mM) Cu^{2+}, Hg^{2+}, Fe^{3+} and Mn^{2+} was partially reversed by the metal chelator, EDTA. The percent relative activities of Cu^{2+}, Hg^{2+}, Fe^{3+} and Mn^{2+} in the presence of EDTA were 87%, 52%, 90% and 85%, respectively. The maltooligosaccharide-forming amylase in the present study was more resistant to heavy metals like

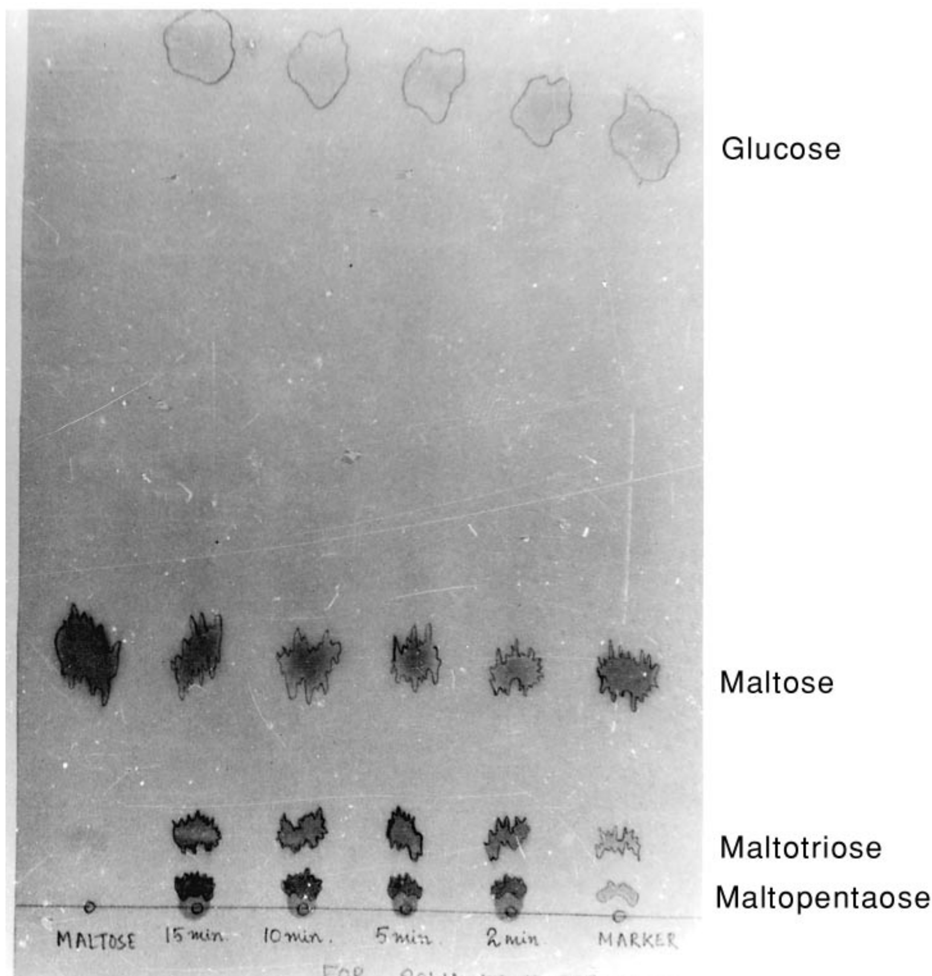

Figure 10 Paper chromatogram of the starch hydrolysate. The maltooligosaccharide markers (maltopentaose, maltotriose, maltose and glucose) were procured from Sigma.

Fe^{3+} and Cu^{2+}, compared to a similar enzyme which showed only 46.8% and no activity in the presence of Fe^{3+} and Cu^{2+}, respectively, at a low metal ion concentration of 1 mM [17].

Product of hydrolysis

Figure 10 shows a paper chromatogram of products produced by the action of this amylase on starch: maltopentaose, maltotetraose, maltose and glucose.

The composition of the hydrolysis products was about 31% maltopentaose, 25% maltotetraose, 1% maltotriose and 30% maltose. The action pattern of the enzyme from the present strain was different from that of *B. circulans* MG-4. The sugar composition of hydrolysis products from liquefied starch for the latter was maltotetraose, maltotriose, maltose and small amounts of glucose [29].

Conclusion

The reaction conditions for the amylolytic reaction have been optimized using RSM. The significant achievement of the present work is the twofold increase in activity of the enzyme by optimization of the reaction temperature and pH. The amylase from *B. circulans* GRS 313 differed significantly in its properties compared to the earlier reported enzyme; for instance, it was capable of producing maltopentaose and maltotetraose along with maltose and glucose from starch unlike the earlier reported amylase from *B. circulans* MG-4, which did not produce maltopentaose. More significantly, the present enzyme was stable for a long time in the intermediate range of 45–50°C, which is more suitable for the purpose of retarding staling. As the maltooligosaccharide-forming amylases that have been reported earlier were not stable above 40°C, they cannot be used for extended application. Also, the thermostable amylases from *Bacillus* strains may survive the oven temperature and cause overhydrolysis of starch, resulting in guminess of the baked products. Thus, the present amylase from *B. circulans* GRS 313 merits consideration as a potential antistaling agent in the baking industry.

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