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ORIGINAL ARTICLE

Production of thermostable, Ca^{+2}-independent, maltose producing α-amylase by *Streptomyces* sp. MSC702 (MTCC 10772) in submerged fermentation using agro-residues as sole carbon source

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Abstract The purpose of this study was to determine the influence of growth conditions and medium composition on the thermostable, Ca^{+2}-independent, maltose producing α-amylase production in submerged fermentation (SmF) by *Streptomyces* sp. MSC702—a strain newly isolated from mushroom compost. Production of α-amylase enzyme by a *Streptomyces* strain was detected on semi-synthetic media containing rice bran and wheat bran in a 1:2 ratio as the sole carbon source. The effect on α-amylase production of different medium ingredients and additives, such as NaCl, additional carbon sources, nitrogen sources, metal salts, nucleotides, phytohormones, surfactants and other chemicals, was examined. NaCl played no notable role in the biosynthesis of α-amylase, with the strain being able to grow at 14% (w/v) NaCl with ~76% α-amylase activity. Among the different cations tested, Hg^{2+} showed no inhibitory effect on α-amylase production. The best combination of physical parameters for the maximum production of α-amylase was a temperature of 50°C, pH 7.0 and an incubation period of 48 h. The best combination of chemical parameters with rice bran 1.0% (w/v) and wheat bran 2.0% (w/v) was dipotassium hydrogen phosphate 0.1% (w/v), magnesium sulfate 0.1% (w/v) with D-inositol (as additional carbon source) 1.0% (w/v), peptone (as nitrogen source) 0.1% (w/v), manganese sulphate (as additional metal ion) 0.05% (w/v) and Tween-80 (as surfactant) 1.0% (v/v). With the application of these culture conditions, α-amylase production increased by ~1.42 fold.

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Introduction

Amylases are among the most important hydrolytic enzymes for all starch-based industries. The commercialization of amylases is quite old, with first documented use in 1984 as a pharmaceutical aid for the treatment of digestive disorders. Nowadays, amylases find application in many industrial processes, such as in the bread and baking industry (Ammar et al. 2002; Gupta et al. 2003), detergents (Kottwitz et al. 1994), textile desizing (Hendriksen et al. 1999) and in the paper industry (Bruinenberg et al. 1996) for the hydrolysis of starch. In this context, microbial amylases have completely replaced chemical hydrolysis in the starch processing industry. They can also be of potential use in the pharmaceutical and fine chemical industries for conversion of starch into sugar syrups containing oligosaccharides, maltose, and glucose (Pandey et al. 2000). Recently, microbial amylases have found applications in the production of bioethanol, where they are used to release sugars from stored starches.

In the starch processing industry, there are many advantages of using thermostable enzymes, such as increased reaction rate and decreased contamination risk through the use of high temperatures (Kaur and Satyanarayana 2004; Kelly et al. 1986). Thermophilic microorganisms are the most important sources for thermostable amylase production. Maltose-forming α-amylases have been isolated from both bacterial and fungal sources (Collins et al. 1993). Maltose is produced by α-amylases of thermophilic actinomycetes, including *Thermoactinomyces* sp. no. 15 (Obi and Odibo 1984), *Streptomyces thermoviolaceus* (Goldberg and

Edwards 1990), *Thermomonospora curvata* (Collins et al. 1993) and *Streptomyces* sp. (Ammar et al. 2002). However, starch degradation by almost all thermostable amylases involves the synchronous production of appreciable levels of glucose (Doyle et al. 1989). The global market for enzymes was about US $2 billion in 2004. It is expected to have an average annual growth rate of 3.3% (Sivaramakrishnan et al. 2006). Amylolytic enzymes alone account for almost US $225 million (Walsh 2002). Due to the increasing demand for amylase enzymes in various industries, there is enormous interest in developing amylases with better properties, such as raw-starch-degrading amylases suitable for industrial applications, and in developing cost-effective production techniques (Burhan et al. 2003).

There is no general defined medium for extracellular amylase production by different microbial strains (Pandey et al. 2000). Every microorganism has its own peculiar physio-chemical and nutritional requirements for extracellular amylase production. It is well documented that amylase enzyme production by microbes is influenced greatly by medium components, especially carbon and nitrogen sources, minerals and physical factors such as pH, temperature, agitation, dissolved oxygen and inoculum density (Babu and Satyanarayana 1993; Dey et al. 2001; Gigras et al. 2002). In view of the commercial utility of the enzyme, formulation of a cost-effective medium becomes a primary concern. This need is manifested in the present interest to produce α-amylase by a thermophilic actinomycete on media based on agro-residues (rice bran and wheat bran). The moderate thermostability and Ca^{2+} requirement of amylases limit their industrial potential. This investigation was therefore carried out to optimize the production of high maltose-forming, thermostable and Ca^{2+}-independent α-amylase by *Streptomyces* sp. MSC702 in low-cost fermentation medium containing rice bran and wheat bran as the sole carbon source.

Materials and methods

All medium ingredients and reagent chemicals were of analytical grade and were procured from E-Merck, Hi-Media and Qualigen Chemicals, India, Ltd. Fresh rice bran and wheat bran, used as the sole carbon source, were obtained from a local market in Kanpur, India.

Screening and maintenance of α-amylase-producing thermophilic actinomycete

Streptomyces sp. MSC702 was isolated from mushroom compost collected from the Mushroom Research and Cultivation Center, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, India. A total of seven thermophilic actinomycete isolates were screened for α-amylase production on M medium (Obi and Odibo 1984), containing (% w/v) soluble starch 1.0, K_2HPO_4 0.1, $(NH_4)_2SO_4$ 0.1, NaCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.1, pH 7.0 at 45°C. M medium was modified with 1% trace metal salt solution (Techapun et al. 2002), and amylolytic isolates were screened out by flooding the agar plates with 0.001 M iodine solution (Obi and Odibo 1984). Isolates with high clearing zone were grown in liquid broth and the amount of α-amylase production was determined in culture filtrates. Subsequently, the maximum α-amylase producing strain was selected and cultivated in modified M medium agar slants for 2–3 days at 50°C and stored at 4°C for further studies. All experiments were carried out in triplicate and the data presented are average values.

Strain MSC702 was deposited in the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India with the accession number MTCC 10772.

Inoculum preparation

The inoculum was prepared by adding 10 ml sterile distilled water to a 2 to 3-day-old culture slant and a cell suspension was made with the help of a sterile loop. Cell suspension ($1.9–2.2 \times 10^8$ CFU/ml) was used as the inoculum for α-amylase production.

Enzyme production in submerged fermentation

α-Amylase production in submerged fermentation (SmF) was carried out using basal medium containing (% w/v) rice bran 1.0, wheat bran 2.0, K_2HPO_4 0.1, $(NH_4)_2SO_4$ 0.1, NaCl 0.1 and $MgSO_4 \cdot 7H_2O$ 0.1 at pH 7.0. Production medium (100 ml) was placed in an Erlenmeyer flask (250 ml) and autoclaved at 121°C for 20 min and cooled. The medium was inoculated with 1% inoculum and incubated at 50°C for 48 h. Samples were harvested by filtering through Whatman filter papers 1 (qualitative circles, 125 mm diameter) and centrifuged at 5,000 *g* for 20 min at 4°C; the cell-free supernatant (crude enzyme) was used for α-amylase assay.

Growth kinetics

The growth of the microorganism in relation to α-amylase production (12–96 h) was studied in medium containing 1% starch, pH 7.0 incubated at 50°C. At an interval of 12 h, the growth of the microorganism was monitored by recording optical density at 610 nm.

Effect of temperature and pH

Temperatures ranging from 45 to 70°C and pH values in the range of 5.0 to 10.0 were examined for their effect on

α-amylase production by the strain using basal medium. The pH of the medium was adjusted using 1 *N* HCl or 1 *N* NaOH.

Effect of NaCl

Effect of NaCl on α-amylase production was studied by varying the concentrations of NaCl (0–14% w/v) added to basal medium containing rice bran and wheat bran in 1:2 ratio, pH 7.0 incubated at 50°C for 48 h. α-Amylase activity was expressed as percentage relative activity.

Effect of additional carbon sources

Various carbon sources, such as monosaccharides (arabinose, xylose, glucose, fructose, galactose), disaccharides (sucrose, maltose, lactose, cellobiose), trisaccharides (raffinose), polysaccharides (starch, cellulose), deoxy sugar (L-rhamnose) and polyhydroxy alcohols (D-sorbitol, D-mannitol, D-inositol) were evaluated for their effect on α-amylase production by adding 1% (w/v) in basal medium containing rice bran and wheat bran in 1:2 ratio.

Effect of nitrogen sources

In the basal medium, ammonium sulphate (0.1% w/v) was replaced by different inorganic and organic nitrogen sources with the same percentage. Different nitrogen sources examined were $NaNO_3$, NH_4NO_3, $(NH_4)_2HPO_4$, urea, thiourea, peptone, yeast extract, tryptose and casein.

Effect of different additives

In the basal medium, effect of metal salts, nucleotides, phytohormones, surfactants, EDTA and gallic acid on α-amylase production was determined. α-Amylase activity was determined as percentage relative activity as compared to the control (without additives), which was considered as having 100% relative activity.

Repression study

Repression of enzyme secretion by glucose and maltose was studied by incorporating different concentrations of maltose and glucose (0.5–2.5% w/v) into the basal medium. α-Amylase activity was expressed as percentage relative activity.

Enzyme assay

α-Amylase activity was estimated by analyses of reducing sugar released during hydrolysis of 1.0% (w/v) starch in 0.1 M phosphate buffer (pH 7.0) by enzyme (cell-free supernatant) incubated at 50°C for 10 min. The amount of reducing sugar level released in the mixture was determined by the dinitrosalicylic acid (DNS) method (Miller 1959). Absorbance at 550 nm was recorded by using UV-visible spectrophotometer (UV-1700 Pharmaspec Shimadzu) and activity was calculated from a standard curve using maltose as the standard. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1 μmol reducing sugar as maltose per minute under standard assay conditions.

Raw starch hydrolysis

The action of the crude α-amylase on soluble potato starch or raw starch granules of wheat, corn, barley, rice, trapa and buckwheat was examined. The reaction mixture consisted of 5.0% soluble potato starch or raw starch granules (flour) in 0.1 M phosphate buffer (pH 7.0), and was incubated in a shaking water bath at 50°C with 1 U/ml enzyme for 2 h. The reaction mixture was centrifuged at 5,000 *g* for 20 min. The reducing sugar in the resulting supernatant phase was determined by the DNS method (Miller 1959) with maltose as standard. For acid hydrolysis, the reaction mixture consisted of 5.0% of soluble starch or raw starch granules, and was incubated in a shaking water bath at 100°C with 6.0 M HCl for 2 h.

The extent of hydrolysis of soluble potato starch and raw starch (R_h) was defined by the formula:

$$R_h = \frac{\text{Amount of sugar produced by the enzyme hydrolysis}}{\text{Amount of substrate before the reaction}} \times 100$$

The degree of hydrolysis (%) of soluble and raw starch was defined by the formula:

$$\text{Degree of the hydrolysis (\%)} = \frac{\text{Reducing sugar produced by the enzyme hydrolysis}}{\text{Reducing sugar produced by the acid hydrolysis}} \times 100$$

Results and discussion

The use of agricultural biomass as carbon source in culture media leads to reduction in the cost of enzyme production (Tengerdy and Szakacs 2003). Different sources of lignocellulose, such as wheat bran, rice husk and sugarcane bagasse have been utilized to produce various extracellular enzymes (Parajo et al. 1998; Pessoa et al. 1997). Rice bran and wheat bran are high-volume low-value agro-industrial residues and therefore represent feasible growth substrates for economical fermentation processes with more than 40% sugar content (Shatta et al. 1990) (Table 1).

Thermophilic actinomycetes are well known to synthesize many secondary metabolites and thermostable extra-

Table 1 Chemical analysis of rice bran and wheat bran used as sole carbon source (Shatta et al. 1990)

Raw material	Moisture (%)	Ash (%)	Total sugars (%)	Total nitrogen (%)	Protein (%)
Rice bran	8.45	10.80	43.14	1.79	11.20
Wheat bran	12.00	10.00	40.80	1.78	11.00

cellular enzymes, including amylase, cellulase and xylanase, that have potential applications in various industries (Haki and Rakshit 2003).These Gram positive bacteria with high G+C content, growing above ambient temperature (50–60°C) can be isolated from compost and overheated plant materials such as hay and bagasse. Here, we report the production of thermostable, Ca^{+2}-independent, maltose-producing α-amylase by a new species of *Streptomyces*, a thermophilic actinomycete designated as MSC702 isolated from mushroom compost. We applied classical approach (single factor variation, keeping other factors constant) for optimization of culture parameters to enhance α-amylase production by *Streptomyces* sp. MSC702.

Growth characteristics and effect of incubation period on α-amylase production

Strain MSC702 shows good growth in the temperature range 45–60°C in 48 h in basal medium. However, the strain was able to grow at 65–70°C with comparatively less biomass in basal medium. Good growth of the strain was observed at pH 6.5–7.5, with the ability to grow under highly alkali conditions (pH 10.0). Thus strain MSC702 exhibited a highly thermophilic as well as an alkaliphilic nature.

Most actinomycetes species are slow growing. Enzyme production starts in early log phase but there is a drastic increase in production of enzyme at late growth phases (Chakraborty et al. 2009). In constrast, strain MSC702 is a fast-growing thermophilic actinomycete, and showed maximum α-amylase production (276.34 U/ml, 48 h) in early log phase with a drastic decrease in production of enzyme (232.56 U/ml, 72 h) in late growth phase or early stationary phase (Fig. 1). The production of α-amylase by strain MSC702 began after 12 h and reached a peak of 276.34 U/ml at 48 h, showing that α-amylase production was independent of the growth phase. Similar reports on α-amylase were reported on *Streptomyces albidoflavus* (Narayana and Vijayalakshmi 2008) and *Streptomyces erumpens* MTCC 7317 (Kar and Ray 2008).

Effect of temperature and pH

The impact of temperature and pH on α-amylase production is depicted in Figs. 2 and 3. Strain MSC702 was

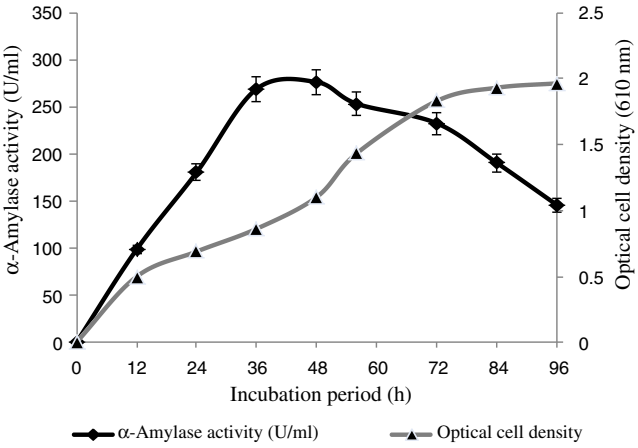

Fig. 1 Growth kinetics of strain MSC702 with reference to α-amylase production. The growth of the microorganism (12–96 h) was studied in medium containing 1% starch, pH 7.0, and incubated at 50°C

capable of producing α-amylase in the range of 45–70°C with maximum production at 50°C (560.78 U/ml) while retaining ~72% and ~35% α-amylase synthesis at 60°C and 65°C, respectively (Fig. 2). Many thermophilic actinomycetes, such as *Thermomonospora curvata* and *Thermomonospora fusca* (Busch and Stutzenberger 1997), *Streptomyces* sp. IMD 267 (McMahon et al. 1997), *Streptomyces* sp. D1 (Chakraborty et al. 2009) and *S. erumpens* MTCC 7317 (Kar and Ray 2008) have been reported to produce α-amylase at the same temperature range.

The present study showed an optimum pH range of 6.5–7.5 for α-amylase biosynthesis by strain MSC702, with maximum production at pH 7.0 (578.67 U/ml) (Fig. 3). Similarly, Kuo and Hartman (1966) found that *Thermoactinomyces vulgaris* synthesized amylase most rapidly at pH values ranging from 6.5 and 7.5, and that amylase inactivation occurred rapidly if the pH rose above 7.5. Amylase production by *Streptomyces aureofaciens* 77 (Shatta et al. 1990), *S. erumpens* MTCC 7317 (Kar and

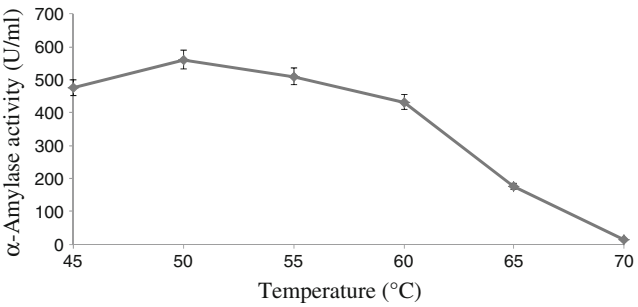

Fig. 2 Effect of different temperature (45 to 70°C) on α-amylase production by strain MSC702 was examined using basal medium containing rice bran and wheat bran in 1:2 ratio (pH 7.0), incubation period 48 h

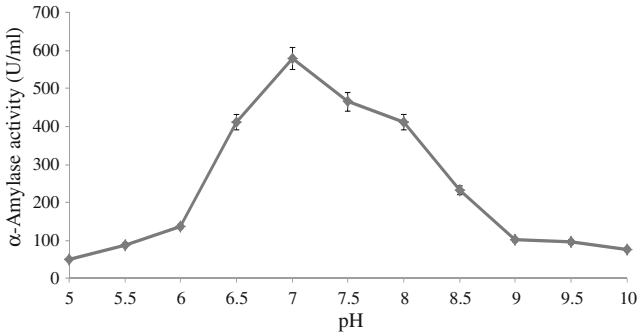

Fig. 3 Effect of different pH (5.0–10.0) on α-amylase production by strain MSC702 was examined using basal medium containing rice bran and wheat bran in 1:2 ratio, incubated at 50°C for 48 h

Ray 2008) and *S. rimosus* (Yang and Wang 1999) increased gradually as initial pH values ascend from 5.0 to 7.0.

Effect of NaCl

Strain MSC702 was found to be highly halophilic in nature. The results obtained in this study revealed that the presence or absence of NaCl in basal medium has no effect on α-amylase production by this strain. It retained 100% and 80% of its maximum activity in the presence of 0–6% and 8–12% NaCl, respectively. Maximum α-amylase production was obtained in the absence of NaCl while the enzyme retained almost 75.98% of its activity in presence of 14% (w/v) of NaCl. Chakraborty et al. (2009) also found that *Streptomyces* sp. D1 retained 80% of its amylase activity on 12% (w/v) NaCl.

Effect of additional carbon sources

The effect of different carbon sources on α-amylase production is shown in Fig. 4. Among all the tested carbon sources used in basal medium, polyhydroxy alcohols like D-inositol and D-sorbitol were found to be best inducers for α-amylase production, followed by monosaccharides like glucose and galactose and polysaccharides like starch. The presence of D-mannitol and all tested disaccharides did not affect α-amylase synthesis markedly. A deoxy sugar (L-rhamnose) strongly suppressed α-amylase production. Among monosaccharides, arabinose, xylose and fructose inhibited α-amylase production. D-Inositol induced α-amylase production (665.37 U/ml) more than other carbon sources. Similar results were reported by Srivastava and Baruah (1986), who also observed the positive effect of inositol on amylase production by *Bacillus stearothermophilus*. Kuo and Hartman (1966) showed that *Thermoactinomyces vulgaris* produced best yields of α-amylase in the presence of starch or maltose, while in the present study the presence of maltose in the production medium suppressed enzyme synthesis. Hamilton et al. (1999) reported highest α-amylase production by *Bacillus* sp. IMD 435 in the presence of lactose. In contrast, lactose suppressed α-amylase production by strain MSC702.

The results revealed that D-inositol induced both growth and α-amylase production. Available evidence also favors the view that inositol may be used in the formation of an actual building unit in the structure of the cell, and it is utilized as a carbon source for growth by strain MSC702. Strain were found to absorb considerable amounts of D-inositol during growth and this affected α-amylase production in a quantity proportional to the amount of growth of the organism; when the external supply of D-inositol was exhausted there was an immediate slowing of growth rate and α-amylase production compared with that on an D-inositol-free medium (control). Lane and Williams (1948) stated that highly purified pancreatic amylase contains an exceptionally high proportion of meso-inositol. Contrasting results were presented by Zahner and Ettlinger (1957) in which the presence of myo-inositol neither affected mycelial weight nor was utilized as a carbon source for growth by *Streptomyces griseus*. Apart from growth and α-amylase production, D-inositol may be used in combination with arginine to enhance streptomycin

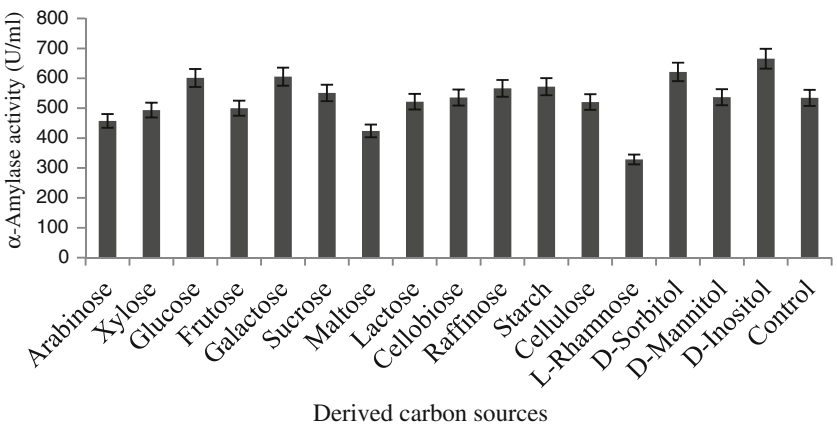

Fig. 4 The effect of additional derived carbon sources on α-amylase production was evaluated by adding 1% (w/v) of various carbon sources to basal medium containing rice bran and wheat bran in 1:2 ratio, pH 7.0 incubated at 50°C for 48 h

production, and it is also utilized in the biosynthesis of mycothiol (MSH). MSH—a major intracellular thiol (Newton et al. 1995) present in the genus *Streptomyces*—contains inositol as a sugar moiety. The enzyme plays important roles in maintaining the redox balance within the cell, and protects the cell against nitrosative stress and toxins (Rawat and Av-Gay 2007).

Effect of nitrogen sources

The effect of different nitrogen sources on α-amylase production by strain MSC702 is presented in Table 2. All inorganic and organic nitrogen sources used in the present study affected α-amylase production positively. The medium amended with peptone produced maximum α-amylase activity, followed by ammonium sulfate, ammonium nitrate and diammonium hydrogen phosphate. Narayana and Vijayalakshmi (2008) reported similar results for α-amylase production by *Streptomyces albidoflavus*. Vidal et al. (1995) and Upton and Fogarty (1977) proved in their research that peptone is the best nitrogen source for amylase production.

Effect of different additives

α-Amylase production in the presence of different metal ions was investigated (Table 3). Some of metal ions were stimulating, some showed no effect, while a few had an inhibitory effect on α-amylase production. Table 3 summarizes the effect on α-amylase production by *Streptomyces* sp. MSC702 of these different additives. Among the tested divalent cations, α-amylase production was stimulated by the presence of Mn^{2+}, Mg^{2+} and Co^{2+} ions. Other divalent cations such Pb^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mo^{2+}, Ca^{2+} and Sn^{2+} inhibited α-amylase production, while Hg^{2+} showed no effect on α-amylase production. Hg^{2+} has been shown to have a potent inhibitory effect on growth of microorganisms and production of α-amylase from *Chromohalobacter* sp. TVSP 101 (Prakash et al. 2009) and *Aspergillus niger* (Hernandez et al. 2006), whereas it had no effect at all on enzyme production of *Streptomyces* sp. MSC702.

All the univalent and trivalent cations (K^+, Ag^+, Al^{3+}, Ag^{3+} and Cr^{3+}) tested showed an inhibitory effect on α-amylase production. Similar to the present study, Mn^{2+},

Table 2 Effect of different nitrogen sources on α-amylase production[a]

Nitrogen source (0.1%)	α-Amylase enzyme activity (U/ml)
$NaNO_3$	411.61
$(NH_4)_2SO_4$	573.74
NH_4NO_3	566.36
$(NH_4)_2HPO_4$	499.19
Urea	423.46
Thiourea	424.33
Peptone	584.38
Yeast extract	368.66
Tryptose	370.27
Casein	367.07
Control[b]	310.32

[a] α-amylase production was carried out in basal medium containing rice bran and wheat bran in 1:2 ratio, pH 7.0 incubated at 50°C for 48 h

[b] Control—medium without nitrogen source

Table 3 Effect of various metal salts, nucleotides, phytohormones, surfactants and other chemicals on α-amylase production

Metal ions (0.05% w/v)	Relative α-amylase activity (%)
KCl	80.85
AgCl	72.12
$AgNO_3$	66.58
$Pb(NO_3)_2$	63.78
CrO_3	78.19
$MnSO_4 \cdot H_2O$	118.03
$MgSO_4 \cdot 7H_2O$	116.88
$FeSO_4 \cdot 7H_2O$	65.41
$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	71.14
$CoSO_4 \cdot 7H_2O$	107.00
$CoCl_2$	115.61
$CuSO_4$	92.7
$ZnSO_4$	90.82
$ZnCl_2$	83.07
$(NH_4)_6Mo_7O_{24}$	68.14
$CaCl_2$	76.75
$HgCl_2$	100.01
$SnCl_2$	68.28
$AlCl_3$	63.33
Nucleotides/ phytohormones (0.05% w/v)	
Uracil	79.45
Adenine	81.03
Xanthine	89.36
IAA	73.21
NAA	81.09
GA_3	79.07
Surfactants/Chemicals	
Triton X-100 (1% v/v)	67.17
Tween 80 (1% v/v)	111.54
Sodium lauryl sulphate (1% w/v)	59.67
Glycerol (1% v/v)	90.03
EDTA (0.05% w/v)	89.05
2,3,5 T (Gallic acid) (0.05% w/v)	102.86
Control[a]	100

[a] Control—medium without any additives

Mg^{2+} and Co^{2+} ions promoted α-amylase production in *Bacillus* sp. I-3 (Goyal et al. 2005), *Vibrio* sp. (Najafi and Kembhavi 2005) and *Bacillus* sp. L1711 (Bernharsdotter et al. 2005), respectively. In contrast, Mn^{2+}, Mg^{2+} and Co^{2+} ions inhibited amylase production in *Aspergillus oryzae* EI 212 (Kundu et al. 1973), *Bacillus* sp. CRP (Wu et al. 1999) and *Streptomyces* sp. IMD 2679 (McMahon et al. 1997), respectively. K^{+}, Fe^{2+} and Mo^{2+} showed no effect on α-amylase production by *A. oryzae* EI 212, while Ca^{2+} inhibited its production (Kundu et al. 1973). Mg^{2+} positively affected α-amylase production in *Nocardiopsis aegyptia* (Abou-Elela et al. 2009). Srivastava and Baruah (1986) also reported the inhibitory effect of trivalent ions on amylase production by *Bacillus stearothermophilus*. Zn^{2+} was reported to inhibit thermostable α-amylases from a thermophilic *Bacillus* sp., suggesting that inhibition with the ion determined the thermostability of the enzyme. The presence of Zn^{2+} decreased the activity of *Bacillus* sp. ANT-6 enzyme, indicating its high thermostability (Burhan et al. 2003). Ozlem et al. (2005) reported the inhibitory effect of Zn^{2+} and stimulatory effect of Mn^{2+} on α-amylase production by *Bacillus* sp. K-12. The present study on *Streptomyces* sp. MSC702 showed a remarkable degree of agreement with these reports.

Different concentrations of $CaCl_2$ (0–50 mg) were amended in basal medium containing rice bran and wheat bran in 1:2 ratio for characterization of α-amylase. With increased concentration of Ca^{2+} in basal medium, α-amylase production was proportionally inhibited. In the presence of 50 mg/ml Ca^{2+} in basal medium, α-amylase activity corresponded to around 53.38% compared to the control experiment. For production and stability of α-amylase by *Bacillus* spp., addition of Ca^{2+} ions is often necessary (Egas et al. 1998). However, in this study, upon increasing the Ca^{2+} concentration in medium, α-amylase production was suppressed. Thus, α-amylase production by *Streptomyces* sp. MSC702 was Ca^{2+}-independent. Similar results were reported for *S. erumpens* MTCC 7317 (Kar and Ray 2008) and *B. thermooleovorans* NP54 (Malhotra et al. 2000). Calcium-independent amylases merit consideration for starch liquefaction, especially in the manufacture of fructose syrup, where calcium is a known inhibitor of glucose isomerase (Tonkova 2006).

All the nucleotides (uracil, adenine and xanthine) and phytohormones (IAA, NAA and GA_3) tested inhibited α-amylase production (Table 3). Srivastava and Baruah (1986) also reported the inhibitory effect of several nucleotides (ADP, GTP, guanine, cytosine, adenine, uracil, xanthine and NADP) on amylase production by *Bacillus stearothermophilus*. Very scarce information about the effect of plant-derived active compounds on *Streptomyces* sp. physiology is available. The influence of phytohormones on different aspects of life cycle and morphology of pathogenic and saprophytic microorganisms has been the object of several studies (Ishii et al. 1996). Gemishev et al. (2005) reported the positive effect of phytohormones on relative endo-1.4-β-endoglucanase activity in cultures of *Trichoderma reesei*. To the best of our knowledge, however, there has been no report on the use of phytohormones for production of α-amylases by *Streptomyces* sp. in previous studies.

In the present study, α-amylase production decreased drastically in the presence of sodium lauryl sulphate (SLS) (0.05% w/v) and Triton X-100 (1% v/v), while glycerol (1% v/v) reduced enzyme production slightly (Table 3). Tween 80 (1% v/v), on the other hand, stimulated α-amylase production up to ~12%. Kar and Ray (2008) also reported the inhibitory effect of SLS (0.02% w/v) and Triton X-100 (0.02% v/v) on α-amylase production by *S. erumpens* MTCC 7317. Increased production of α-amylase in the presence of Tween 80 has been reported on *Thermomyces lanuginosus* (Arnesen et al. 1998). Kar and Ray (2008) and Suzuki et al. (2000) reported that growth and biochemical production of actinomycetes are stimulated by glycerol. In contrast, our results revealed the inhibitory effect of glycerol on α-amylase production by *Streptomyces* sp. MSC702. It appears that the same surfactant may stimulate enzyme production in one species and inhibit it in another.

Among other tested chemicals, the chelating agent EDTA (0.05% w/v) reduced α-amylase production, whereas gallic acid (0.05% w/v) induced α-amylase production up to 3% over the control (Table 3). EDTA showed an inhibitory effect on α-amylase production in many microorganisms, such as *Bacillus* sp. K-12 (Ozlem et al. 2005). According to our results, and those of previous studies, the inhibitory effect of the chelating agent EDTA, which binds metal ions, demonstrates the ion requirement of amylase (Ozlem et al. 2005). Gallic acid is a trihydroxybenzoic acid —a type of organic (phenolic) acid—that acts as an antioxidant and helps to protect cells against oxidative damage. The effect of polyphenols on the production of different enzymes has also been studied previously (Arrieta-Escobar and Belin 1982; Costa et al. 2008), but there have been no reports of gallic acid as a media ingredient for α-amylase production. To evaluate the effect of polyphenols on α-amylase production, gallic acid was chosen as the molecule model for this class of compounds, and the results indicated that it may be a good stimulator for α-amylase production by *Streptomyces* sp. MSC702.

Repression study

Strong catabolic repression by maltose was observed (Fig. 5). α-Amylase production decreased drastically with increase in maltose concentration from 0.5 to 2.0% (w/v).

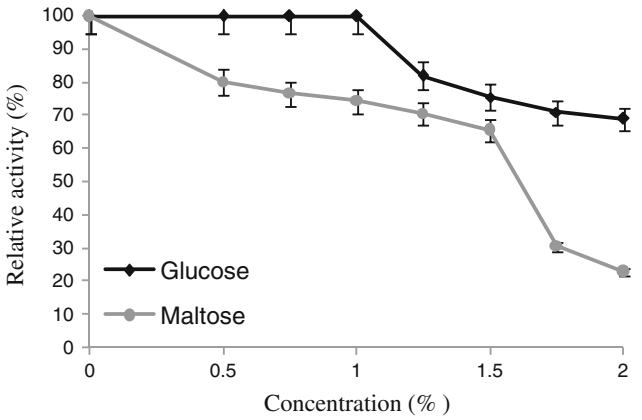

Fig. 5 Effect of different concentration (0.5–2.5% w/v) of glucose and maltose on α-amylase production on basal medium containing rice bran and wheat bran in 1:2 ratio, pH 7.0 incubated at 50°C for 48 h

At low concentrations, glucose had no effect on α-amylase biosynthesis. The results showed that, in comparison to maltose, glucose was required in high amounts to repress α-amylase biosynthesis. The inability of glucose in comparison to maltose to repress α-amylase biosynthesis during growth of *Streptomyces* sp. MSC702 on agro-residues may be due to the high affinity for maltose over glucose. Similar results were obtained by Busch and Stutzenberger (1997) working on *Thermomonospora fusca*. They found that glucose was unable to repress amylase biosynthesis during growth of *T. fusca* on starch. Saxena et al. (2007) also observed same results on *Bacillus* sp. PN5. The results obtained in the present study are supported by the research of Bernier and Stutzenberger (1987) and Polizeli et al. (1996). These latter authors concluded that, unlike other microbes, bacterial and fungal isolates from high temperature composting environments may possess resistance to glucose repression of exoenzyme biosynthesis as part of their adaptation to growth on recalcitrant, heterogeneous mixed polysaccharides.

Raw starch hydrolysis

The raw starch digesting potential of *Streptomyces* sp. MSC702 was tested by investigating the extent of hydrolysis and degree of hydrolysis of various raw starch granules (Table 4). It was observed that the α-amylase by *Streptomyces* sp. MSC702 could hydrolyze efficiently different starch granules in a short time (2 h). The extent of hydrolysis at 2 h of soluble potato starch and flours of wheat, corn, barley, rice, trapa and buckwheat were 24.5%, 25.34%, 27.12%, 26.72%, 24.0%, 26.62% and 24.36%, respectively. Maximum digestibility rate was found for raw corn starch and maximum digestibility rate, in comparison to acid hydrolysis, was found for raw barley starch. Liu and Xu (2008) and Hamilton et al. (1999) also reported maximum digestibility rate for corn starch granules by α-amylase from *Bacillus* sp. YX-1 and *Bacillus* sp. IMD 435, respectively. Due to the capacity to digest various raw starch granules, α-amylases obtained from *Streptomyces* sp. MSC702 are economically attractive as they can increase the range of starch sources for direct hydrolysis.

Table 4 Comparison of hydrolysis of various raw starches by the crude α-amylase from *Streptomyces* sp. MSC702

Starch	Extent of hydrolysis (R_h) (%)	Degree of hydrolysis (%)
Soluble potato starch	24.50	34.71
Raw wheat starch	25.34	41.31
Raw corn starch	27.12	37.08
Raw barley starch	26.72	41.73
Raw rice starch	24.0	35.60
Raw trapa starch	26.62	36.67
Raw buckwheat starch	24.36	44.56

Conclusion

The thermostable α-amylase obtained from a newly isolated strain of thermophilic actinomycete, *Streptomyces* sp. MSC702, exhibits a high degree of raw starch digestibility. The results revealed that basal medium containing D-inositol (1% w/v) gave maximum yields of α-amylase (665.37 U/ml) by *Streptomyces* sp. MSC702. From laboratory experiments, initial operating and design conditions for large-scale production units can be obtained. Optimum conditions of temperature, pH and incubation period were 50°C, 7.0 and 48 h, respectively, while the optimum levels of additives in production medium were rice bran 1.0% (w/v), wheat bran 2.0% (w/v), K_2HPO_4 0.1% (w/v), $MgSO_4 \cdot 7H_2O$ 0.1% (w/v) with D-inositol (as additional carbon source) 1.0% (w/v); peptone (as nitrogen source) 0.1% (w/v); $MnSO_4 \cdot H_2O$ (as additional metal ion) 0.05% (w/v) and Tween-80 (as surfactant) 1.0% (v/v). Application of these modified culture conditions increased α-amylase production by ~1.42 fold (maximum activity of 752.42 U/ml) compared to that obtained under conditions previously employed in our laboratory using basal medium (531.23 U/ml at 50°C temperature, pH 7.0, 48-h incubation period). Compared with other α-amylases from strains of *Streptomyces* reported previously (Chakraborty et al. 2009; Narayana and Vijayalakshmi 2008; Ammar et al. 2002; Yang and Wang 1999), the α-amylase in this research

possesses some unique properties, making this strain valuable on an industrial scale.

However, our study is based on the production of α-amylase on agro-residues in SmF. There were very few reports on the production of α-amylases in SmF using agro-residues as sole carbon sources. Our results suggest that the economical utilization of agro-residues for cost-effective production of α-amylase in SmF is possible. α-Amylase production by *Streptomyces* sp. MSC702 is significant as it is Ca^{2+}-ion-independent, which is useful in the confectionary industry, particularly in the making of fructose syrups.

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