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Optimization of Thermostable Alpha-Amylase Production Via Mix Agricultural-Residues and *Bacillus amyloliquefaciens*

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Abstract

This study reports utilization of mixture of wheat and barley bran (1:1) for the production of thermostable alpha-amylase enzyme through a spore former, heat tolerant strain of *Bacillus amyloliquefaciens* in solid state fermentation. Maximum yield of alpha-amylase (252.77 U mL^{-1}) was obtained in following optimized conditions, inoculums size 2 mL (2×10^6 CFU/mL), moisture 80%, pH 7±0.02, NaCl (3%), temperature 38±1°C, incubation for 72 h, maltose (1%) and tryptone (1%). After SSF crude enzyme was purified via ammonium sulfate precipitation, ion exchange and column chromatography by DEAE Cellulose. Purified protein showed a molecular weight of 42 kDa by SDS-PAGE electrophoresis. After purification, purified enzyme was characterized against several enzymes inhibitors such as temperature, NaCl, pH, metal and surfactants. Pure enzyme was highly active over broad temperature (50-70°C), NaCl concentration (0.5-4 M), and pH (6-10) ranges, indicating it's a thermoactive and alkali-stable nature. Moreover, $CaCl_2$, $MnCl_2$, β-mercaptoethanol were found to stimulate the amylase activity, whereas $FeCl_3$, sodium dodecyl sulfate (SDS), $CuCl_3$ and ethylenediaminetetraacetic acid (EDTA) strongly inhibited the enzyme. Moreover, enzyme specificity and thermal stability conformed by degradation of different soluble starch up to 55°C. Therefore, the present study proved that the extracellular alpha-amylase extracted through wheat flour residues by organism *B. amyloliquefaciens* MCCB0075, both have considerable potential for industrial application owing to its properties.

Keywords: amylase, enzyme inhibitor, optimization, solid substrate fermentation, SDS

Introduction

Starch is an important renewable biological resource and agricultural residues like corn, wheat and barley contain around 60-75% starch and all these substrates are generally utilized for solid substrate fermentations for production of different lytic enzymes (Gangadhran *et al.,* 2006; Kunamneni and Singh, 2005). Among all important groups of hydrolytic enzymes, amylases have been also utilized extensively because of their potential application in the biotechnological-based food, detergent, paper, and pharmaceutical industries in India and worldwide (Gangadhran *et al.,* 2006; Li and Xu, 2011) and account for approximately 25% of the total lytic enzyme sales worldwide (Rao *et al.,* 1998). However, most industrial process are carried out under harsh physicochemical conditions, which may not be definitively adjusted to the optimal points required for the activity of the available enzymes; thus, it would be of great importance to develop enzymes that exhibit optimal activities at various ranges of salt concentration, pH, and temperature.

To meet the current largely increased demands of industrially important enzymes, a cost effective substrate and microorganisms becomes the most important sources for enzyme production (Gangadhran *et al.,* 2006; Li and Xu, 2011). Selection of the right organism plays a key role in high yield of desirable enzymes (Pandey 1990). However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey *et al.,* 2000). Nowadays the stability of enzymes becomes a serious problem for the food industries and the major factor which affected the enzyme activity is temperature. Therefore, halotolerant, thermostable, metal tolerant enzymes, which have been produced by microbes, have found a number of commercial applications because of their overall inherent stability (Demirijan *et al.,* 2001; Syed *et al.,* 2009). These enzymes offer robust catalyst alternatives, able to withstand the often relatively harsh conditions of industrial processing. However, starch hydrolyzing activity was widely distributed in species of *Bacillus* and some of them can attack and hydrolyze raw starch granules with the release of dextrose syrups as the predominant product, such enzymes are used for the industrial conversion of raw starch into sugar for fermentation and applied in starch hydrolysis, brewing, baking and detergents industries (Anto *et al.,* 2006). Though, to the best of our knowledge, there are no reports on α-amylase production through mix substrate of wheat and barley residue by an

endospore former *B. amyloliquefaciens* and studies on the effect of enzyme inhibitors on enzyme activity. Therefore, this study focused on the optimization of thermostable alpha-amylase production via mix agricultural-residues and a *Bacillus amyloliquefaciens* strain used in this study.

Materials and methods

Bacterial strain, growth media, substrates and extraction buffers

The strain of *Bacillus amyloliquefaciens* (MCCB0075) was obtained by Microbial Culture Collection Bank, Allahabad Agriculture Institute Deemed University, Allahabad, Uttar Pradesh, India. Stock cultures were grown on nutrient agar (Himedia, India) slant at 37 ± 1 °C for 24 h and maintained on nutrient agar slant at 4 °C. Bran of corn, wheat and barley were obtained from nearest flour mills of Uttar Pradesh, India. These substrates were mixed (1:1) in a flask and screened for their suitability for α-amylase production through SSF on different 2 mL (1×10^6 CFU/mL), moisture 75%, pH 7 ± 0.02, NaCl (3%), temperature 37 ± 1°C and incubation for 72 h. After fermentation different extraction buffers were used for α-amylase enzyme extraction.

Optimization and Enzyme production

Bran is an agriculture residue and it was screened as suitable substrate for α-amylase production and again SSF was carried out by taking 5g of dry substrate and salt solution containing (KH_2PO_4 2 g, NaCl 1 g, NH_4NO_3 10 g, $MgSO_4.7H_2O$ 1 g, H_2O 1L) and distilled water was added to maintain the moisture level in the Erlenmeyer flask (250 mL). Contents of the flasks were mixed properly and autoclaved at 121 °C for 20 min. The physical and nutritional conditions were optimized following 'one-at-a-time' approach to enhance the yield of amylase enzyme. Six different inoculum sizes (0.5, 1, 2, 4, 6 and 8 cfu/ml), seven moisture levels (55, 60, 65, 70, 75, 80, 85 and 90%), eight types of pH of medium (2, 4, 5, 6, 7, 8, 9 and 10), seven concentrations of NaCl (2, 3, 4, 5, 6 ,7 and 8%), six incubation temperatures (30, 37, 40, 45, 50 and 55 °C), five incubation times (24, 48, 72, 96 and 120 h),while nutrient supplementation such as carbon sources [glucose, lactose, maltose and soluble starch (1% w/v)] and nitrogen sources [peptone, yeast extract, tryptone and soyabean meal (1% w/v)] were optimized for amylase production by SSF using *B. amyloliquefaciens MCCB0075*. For each parameter of optimization, three sets of independent experiments were carried out and the average values were reported.

Flasks were maintained in an optimized conditions, inoculums size 2 mL (2×10^6 CFU/mL), moisture 80%, pH 7 ± 0.02, NaCl (3%), temperature 37 ± 1°C, incubation for 72 h, maltose (1%) and tryptone (1%). Crude enzyme was extracted by mixing known quantity of fermented matter with extraction medium like 0.2 M phosphate buffer (pH 7 ± 0.02) and then placed onto rotary shaker at 180 rpm for 1 hour. The suspension was centrifuged at 8000 $\times$ *g* at 4°C for 10 min and supernatant was used for enzyme assay. This experiment was carried out in triplicates.

Analytical procedures

Amylase activity was determined by measuring the release of reducing sugar from soluble starch according to the dinitrosalicilic acid (DNS) method described by Bernfeld (1995). Reaction mixture consisted of 1.25 mL of 1% soluble starch, 0.5 mL of 0.2 M phosphate buffer (pH 7 ± 0.02) and 0.25 mL of crude enzyme extract and incubated at 50 ± 1°C for 10 min. At the end of incubation, 3 mL of DNS reagent was added and the tubes were transferred to a boiling water bath for 5 minutes at 90-95°C. The blank contained 0.75 mL of 0.2 M phosphate buffer (pH 7 ± 0.02) and 1.25 mL of 1% soluble starch. The reducing sugar released was measured at 510 nm in spectrophotometer. One unit (IU) of α-amylase is defined as the amount of enzyme releasing one μmole of glucose equivalent per minute under assay conditions. The specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin in the standard curve.

Purification of α-amylase

Fractionation and Ion exchange chromatography

In the initial purification step, the supernatant of the cell free extract obtained after centrifugation was subjected to protein fractionation by (50% saturation) ammonium sulphate precipitation at 4 °C as described by Green and Hughens (1995), with continuous overnight stirring. The precipitates collected by centrifugation (8000×g for 15 min) were dissolved in 0.1 M phosphate buffer, pH 6.0. The enzyme solution was dialyzed against the same buffer for overnight with several changes to remove the salt and assayed by the method described by Plumer (1978). DEAE-cellulose resin was pretreated and equilibrated in 0.1M phosphate buffer (pH 6 ± 0.2). The slurry was then packed into a column (3×12 cm) and equilibrated further with 0.1M phosphate buffer (pH 6 ± 0.2). The dialysate was layered on the column and eluted into 0.6 mL fractions at a flow rate of 36 mL/h with 200 mL linear salt gradient (0.2-1 M NaCl) in the same buffer. The fractions of 0.6 mL per tube were collected and the fractions given reading at OD_{280} were taken for amylase activity assay, and the active fraction was used for enzyme characterization.

Polyacrylamide gel electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Molecular weights were measured by the method proposed by Weber and Osborn (1969), with the aid of an electrophoresis calibration kit.

Effect of NaCl, pH and temperature on enzyme activity

To determine the effect of NaCl concentrations on enzyme activity, the enzyme assay was performed in the presence of 0-4.5 M NaCl. Moreover, effect of pH on enzyme activity, various pH ranging from 3 to 12 at 37 ± 1 °C for 30 min. The pH of the reaction mixture was varied using different 0.1 M buffers (sodium acetate buffer for pH 3.0-4.0, citrate-phosphate buffer for pH 5.0, phosphate buffer for pH 6.0-7.0, Tris-Cl buffer for

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pH 8.0-9.0, and glycine-NaOH buffer for 10.0-12.0). Enzyme samples were mixed with 0.5 mL of 0.1 M phosphate buffer and 0.25 mL of partially purified α-amylase was incubated at various reaction temperatures ranging from 35 to 90 °C for 30 min. The thermal stability of the enzyme at temperatures 35-90 °C was also carried out by for 1h while aliquots were withdrawn at 10 min interval for enzyme assay. All experiments were carried out in triplicates.

Effect of metal ions and other reagents

The effects of different metal ions on α-amylase activity was determined by the addition of the corresponding ion at a final concentration of 5 mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, Na^{1+}, phosphate buffer, Tween 20, Tween 80, Triton X 100, β-mercaptoehanol, SDS, and EDTA at 37 °C and 45 °C, respectively. This experiment was carried out in triplicates.

Substrate specificity and thermal stability

To determine the substrate specificity and thermal stability, the enzyme assay was performed in reaction mixture containing 1% each of soluble starch, glycogen, amylose, and pullulan in phosphate buffer for 30 min at 37°C, 45 °C and 55 °C temperature for 45 min. This experiment was carried out in triplicates.

Results

SSF optimization, purification and enzyme properties

Production of α-amylase using *B. amyloliquefaciens* was screened with six different residues substrates of corn, wheat and barley bran among all only mixed substrate of wheat and barley has showed maximum production with phosphate buffer (140.65 IU) under SSF (Tab. 1). After screening, optimized conditions (Fig. 1) such as inoculums size 2 mL (2 × 10⁶ CFU/mL), moisture 80%, pH 7±0.02, NaCl (3%), temperature 37±1°C, incubation for 72 h, maltose (1%) and tryptone (1%) were optimized and, in optimized condition 252.77

IU yield of crude enzyme recovered by SSF (Tab. 2). This crude amylase was purified by ammonium sulphate fractionation method and the results are summarized in Tab. 2. After precipitation of the amylase by ammonium sulphate, the precipitate was applied to dialysis by phosphate buffer. Further purification of the enzyme was carried out using DEAE-cellulose gel filtration column and a single peak containing starch-hydrolyzing activity
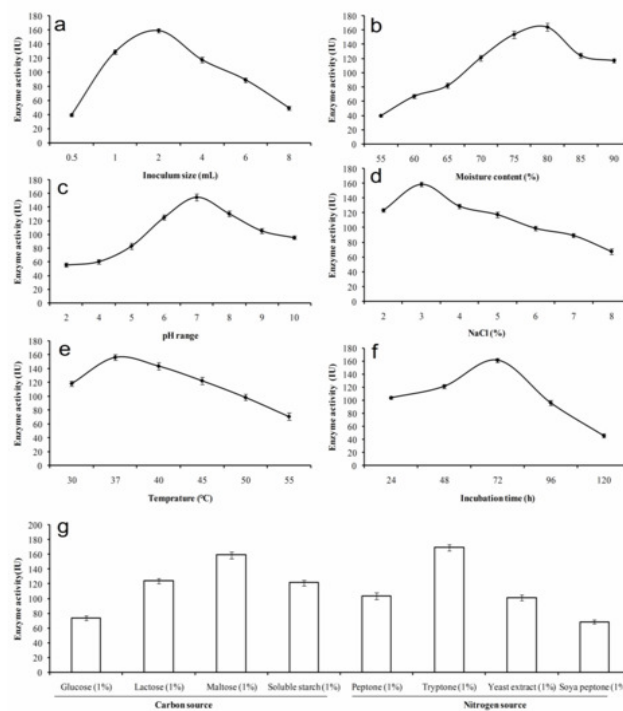

Fig. 1. Optimization of α-amylase production by *Bacillus amyloliquefaciens* MCCB0075 in solid state fermentation through mix substrate of wheat and barley bran using one variable at a time approach. The variables are inoculums size (**a**), moisture content (**b**), pH (**c**), NaCl (**d**), temperature (**e**), incubation time (**f**) and, carbon and nitrogen source (**g**). Enzyme activity was defined as IU detected in the assay. Values are mean ± SE of three independent experiments used in study

Tab. 1. Screening of different agriculture-residues for production of α-amylase enzyme production and extracted by different expiration buffers

Extraction buffers	Barley bran (BB)	Corn bran (CB)	Wheat bran (WB)	BB+CB (1:1)	BB+WB (1:1)	CB+WB (1:1)
Distilled water	57.22±0.58	51.17±0.52	**96.55±0.98**	70.33±0.71	136.38±1.38	62.27±0.63
Phosphate buffer	49.16±0.50	**78.91±0.80**	82.43±0.84	**125.80±1.28**	**140.42±1.43**	**91.51±0.93**
Tween-20	**101.59±1.03**	58.73±0.60	78.91±0.80	86.47±0.88	131.34±1.33	51.67±0.52
Tween-80	44.12±0.45	56.72±0.58	51.67±0.52	114.40±1.16	113.19±1.15	60.25±0.61
TritonX-100	54.20±0.55	47.14±0.48	58.73±0.60	118.94±1.21	111.67±1.13	53.69±0.55

Mean ± SE of three individual replicates were used for study. Different agriculture-residues obtained by local area of Uttar Pradesh and enzymatic activity was quantified based on the amount of reducing sugar (DNS) using starch as substrate.

eluted at approximately 0.6 M NaCl contained starch-hydrolyzing activity and all fractions along with activity are represented in Fig 2. Maximum activity detected in fraction no. 115-120 with 0.267 optical densities at 280 nm. The amylase was purified 11.8-fold with recovery of 64% and specific activity of 45.94 units/mg protein (Tab. 2). The purified enzyme was analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 3, the enzyme appeared as a single band on SDS-PAGE with molecular mass of approximately 42 kDa, in agreement with the size determined by gel filtration.

Effect of NaCl, pH and temperature on the activity

The enzyme assay in the presence of various concentration of NaCl revealed that the enzyme exhibited maximum activity at 0-4.5 M NaCl (Fig. 4a). Also 58.1% and 51.6% of amylase activity were observed at 0 and 3 M NaCl, respectively. The optimum pH for enzyme activity was found to be 7, and the enzyme

retained 56.18% of its maximal activity at pH 10.0 (Fig. 4b). Similarly, maximal activity of enzyme was obtained at on 45 °C and it also retained 69% at 50°C (Fig. 4c). However, the enzyme activity decreased sharply above 55 °C. The purified amylase of *B. amyloliquefaciens* showed maximum activity at 65 °C. At 75 °C after 20 min of incubation, the enzyme showed 36.28% activity. However, prolonged incubation at this temperature decreased the activity. The enzyme was stable between 50 and 70 °C retaining more than 50% activity even after 30 min of incubation. Temperatures above 70 °C had decreased the amylase activity markedly resulting in the complete inactivation of enzyme after 40 min (Fig. 4d).

Influence of metal ions, SDS, β-mercaptoethanol and EDTA

The enzyme activity was significantly activated by metal ions, principally 5 mM Ca^{2+} that increased the activity by 20.51% at 37 °C while at 45 °C activity was slightly inhibited (Tab. 3). In contrast, amylase activity
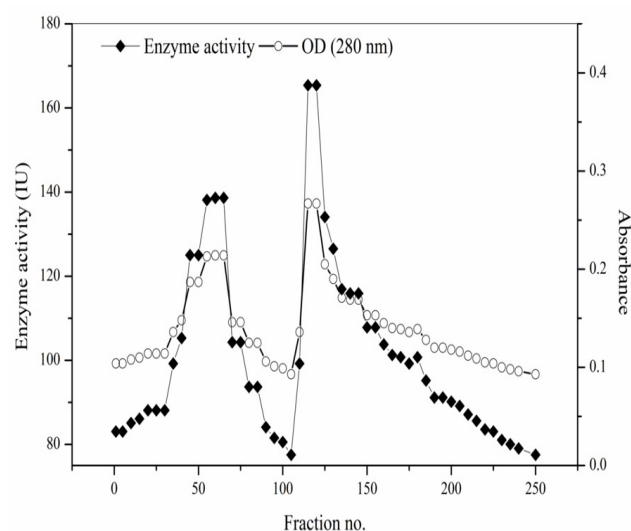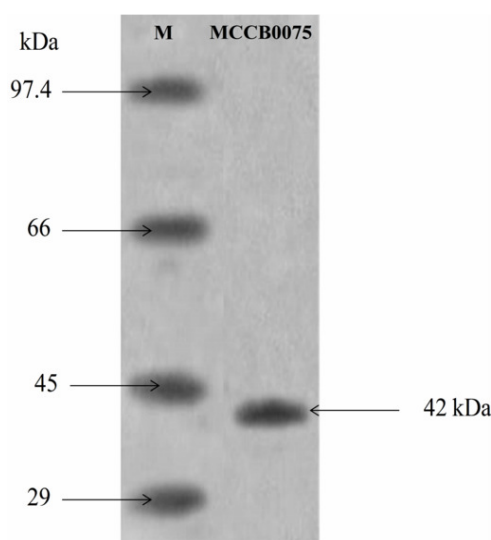

Fig. 2. Profile of the elution of starch hydrolyzing activity of *Bacillus amyloliquefaciens* MCCB0075 by the DEAE-cellulose gel filtration column. Symbols: *circle* (○) absorbance at 280 nm; *black filled square* (♦) amylolytic activity. Enzymatic activity was quantified based on the amount of reducing sugar (DNS) using starch as substrate

Fig. 3. SDS-PAGE analysis of purified amylase of *Bacillus amyloliquefaciens* MCCB0075. Lane 1(**M**) molecular mass markers and lane 2 (**MCCB0075**) DEAE-cellulose gel purified amylase

Tab. 2. Summary of purification steps of the α-amylase from *Bacillus amyloliquefaciens* through wheat and barley mix (1:1)

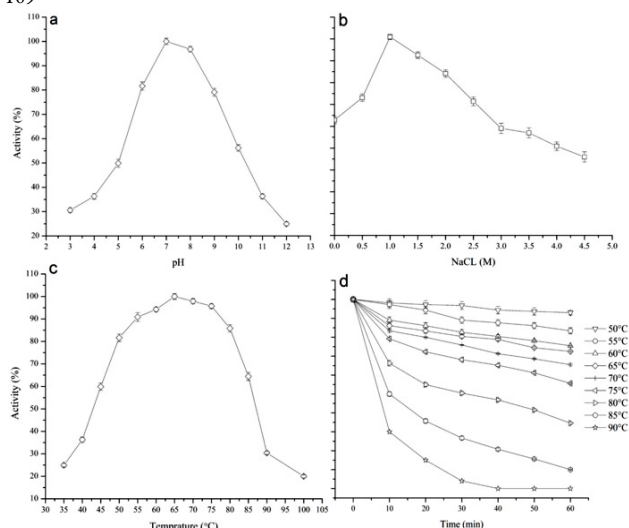| Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude enzyme	252.77	65	3.88	1	100
Ammonium Sulphate Fraction	217.42	26	8.36	2.1	86
Dialysis	190.65	17	11.21	2.89	75
DEAE Cellulose	161.86	3.6	45.94	11.8	64

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Fig. 4. Effect of NaCl (**a**), pH (**b**) and temperatures (**c** and **d**) on the activity of the amylase. Relative activity was defined as the percentage of maximum activity detected in the assay. Values are mean ± SE of three independent experiments

was inhibited by several metal ions, such as Cu^{2+} and Fe^{3+} at both temperature (37 °C and 45 °C). The effect of different salts of various cations, such as Ca^{2+}, Mn^{2+} and Mg^{2+}, and of other reagents, such as NaCl, and KH_2PO_4 was examined on the activity of the amylase, but the enzyme was only slightly affected by temperature. The effects of some known enzyme inhibitor revealed that EDTA strongly inactivated the amylase, but β-mercaptoethanol was found to enhance the amylase activity (to 14.9% of original activity). Moreover, amylase stability was also studied in the presence of some surfactants and purified enzyme was highly stable in the presence of nonionic surfactants, retaining full activity at 37 °C in the presence of Tween 20, Tween 80, and Triton X-100 and gradually increases the activity at

Tab. 3. Effect of metal ions, SDS, EDTA and β-mercaptoethanol on the activity of the amylase produced from *Bacillus amyloliquefaciens* and mean of three individual replicates were used for this study

Compound	Relative activity (%) (5 mM)	
	37°C	45°C
None	100	100
CaCl2	120.51	107.93
MnCl2	112.15	104.30
FeCl3	46.69	42.78
CuCl2	54.23	50.07
MgCl2	104.05	101.39
NaCl	101.35	98.61
KH2PO4	104.99	102.16
Tween 20	74.09	81.69
Tween 80	87.58	92.79
Triton X 100	87.58	90.01
β-mercaptoethanol	114.90	107.70
SDS	53.12	48.99
EDTA	54.93	50.62

45°C. However, the enzyme lost 52% activity in the presence of SDS (Tab. 3).

Substrate specificity and stability

Substrate specificity analysis of the amylase indicated that soluble starch was hydrolyzed at a higher rate than glycogen and amylase at 37 °C and no activity against pullulan was detected (Fig. 5). Similarly, enzyme stability and specificity was detected up to 55 °C in soluble starch, but decreased in high rate in glycogen and amylase (Fig. 5).

Discussion

Being intrinsically stable and active at high temperature, pH and salt, enzymes have important potential and biotechnological applications. A large number with following optimized conditions: inoculums size 2 mL (2 × 106 CFU/mL), moisture 80%, pH 7±0.02, NaCl (3%), temperature 37±1°C, incubation for 72 h, maltose (1%) and tryptone (1%) all variables were optimized. Similarly, the enhanced enzyme production with wheat bran was also reported by Singh
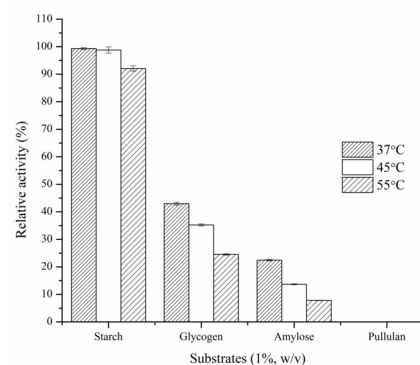

Fig. 5. Substrate specificity of the purified amylase of *Bacillus amyloliquefaciens* MCCB0075 and thermal stability on different temperatures (37-55°C). Relative activity was defined as the percentage of maximum activity detected in the assay. Values are mean ± SE of three independent experiments

of microorganism including bacteria, yeast and fungi produce different groups of enzymes. Selection of a particular strain however, remains a tedious task, especially when commercially competent enzymes, yield are to be achieved. Commercial production of amylases is carried out using both fungal and bacterial cultures. A considerable amount of effort has been dedicated to study of extracellular temperature tolerant enzymes from spore forming *Bacillus*, especially towards use of such enzymes in biotechnological processes (Shafiei *et al.,* 2011). In the present study, Bacillus species are considered to be the most important source of α-amylase and have been used for enzyme production using SSF with bran of three crops (wheat, barley and corn). Among these substrate, mix substrate (1:1) of wheat and barley bran showed maximum α-amylase activity in phosphate buffer. Therefore, wheat and barley mix substrate (1:1) was selected for optimization study

et al., (2009). Wheat and barley bran have been the prime substrate among many processes, which have been developed and have utilized this as a raw material for the production of bulk chemicals and fine products (Pandey 1990; Pandey *et al.*, 2000) and commercial wheat and barley bran contains about 8.5% starch and 9.5% protein (Farid *et al.*, 2011). In the present work, mix substrate of wheat and barley resulted in better amylase production by using *B. amyloliquefaciens* MCCB0075 strain and results are in agreement with those of earlier reports, where wheat bran was the best substrate for amylase production by *B. licheniformis* (Ikram-ul-Haq *et al.*, 2003) and *B. cereus* MTCC 1305 (Anto *et al.*, 2006). Surfactants in the fermentation medium are known to increase the secretion of protein by increasing cell membrane permeability. Therefore, addition of these surfactants (Tween 80) is used for the production of extracellular enzymes. Reports of Arnesen *et al.* (1998) revealed increased α-amylase production by 2-fold in *Thermomyces lanuginosus*. Similar reports of Tweens increasing enzyme yield was observed in the studies of Rao *et al.* (2003) and Gangadhran *et al.* (2006). More the degradation of α-amylase by proteases is one of the reasons for lower activity when distilled water was used as extraction solvents. However, nutrients supplies to the culture also serve as an anchorage for the microbial cells (Farid *et al.*, 2011). The present study findings on the variation in enzyme production with various substrate and extraction solvent is highly comparable with other studies of Gangadhran *et al.* (2006), Sivaramakrishnan *et al.* (2006), Anto *et al.* (2006). This method concluded that phosphate buffer could be utilized as extraction buffer in future.

Moreover, being intrinsically stable and active at high concentration of NaCl, temperature, metal enzymes have potential biotechnological applications, because they are the major enzyme inhibitors. A considerable amount of effort has been dedicated to study of extracellular temperature-tolerant enzymes from mesophilic bacteria, especially towards use of such enzymes in biotechnological processes (Ikram-ul-Haq *et al.*, 2003; Mamo and Gessese, 1999). In this paper we described an α-amylase from a mesophilic bacterium, *B. amyloliquefaciens* MCCB0075. Like many amylolytic enzymes, the amylase from strain MCCB0075 is produced during the exponential growth phase and reached maximum level during the stationary phase (data not shown). According to SDS-PAGE analysis, the amylase is a single polypeptide chain of about 42 kDa. This molecular weight is close to the estimated molecular weight of α-amylases from *Bacillus* sp. YX-1 (54 kDa) (Liu and Xu, 2008) and *B. subtilis* (43 kDa) (Swain *et al.*, 2006). However, the molecular weight of the amylase is slightly lower than that of another two α-amylases with molecular masses of 53 and 76 kDa from thermophilic *Bacillus* sp. strain (Mamo and Gessese, 1999). Studies to determine biochemical properties of the enzyme revealed that, the amylase showed an optimum temperature for activity at 65°C, with loss of activity above 85°C. A temperature optimum of 90°C has been reported for amylases from *B. licheniformis* NCIB6346 (Morgan and

Priest, 1981) and *B. licheniformis* TCRDC-B13 (Bajpai *et al.*, 1989). This property enhances its use in industrial applications that require high temperatures, but favors its application in processes that require complete inactivation of the enzyme with increasing temperature in the process, for example, in the baking industry (Coronado *et al.*, 2000). The optimal pH for the amylase (pH 7) was similar to that reported for amylases from various microorganisms (Bajpai *et al.*, 1989; Liu and Xu, 2008; Sivaramakrishnan *et al.*, 2006; Swain *et al.*, 2006). The amylase is highly active over a board range of salt concentrations (0-3 M) up to 50%. This type of salt tolerance has been observed in α-amylases from *B. dipsosauri* (Deutch, 2002). Amylases with this characteristic may have interesting applications in treatment of high salt waters with starch residues at high temperature.

Enzyme inhibition studies showed that the amylase activity was stimulated by Ca2+ ion and inhibited by EDTA, indicating that the amylase is a metalloenzyme (Sivaramakrishnan *et al.*, 2006). In contrast to another α-amylase from bacterial strain (Li and Yu, 2011), the amylase described here in was stimulated by β-mercaptoethanol. These results suggested that disulfide bonds are not essential for its enzyme activity and could play a role in regulating molecule flexibility to preserve enzyme activity (Carugo *et al.*, 2003). Also, reduction of disulfide bonds in the amylase may lead to changes in the amylase molecule conformation, allowing the active site and the substrate to interact easily (Cinco-Moroyoqui *et al.*, 2008). A similar finding has not been previously reported for other *Bacillus* sp. α-amylases. The effect of various surfactants on the enzyme revealed that, although the amylase was inhibited by 0.5% anionic surfactant (SDS), it was stable in the presence of nonionic surfactants at higher concentrations than those reported for other halophilic α-amylases (Shafiei *et al.*, 2011). Similar findings have not been previously reported for other thermostable amylases. Moreover, the enzyme exhibited remarkable stability towards surfactants, such as SDS and Triton X-100, and may be useful in surfactant industries (Arikan, 2008). To the best of our knowledge, surfactant-stable, thermo active and metal-tolerant α-amylases have not been reported so far through mix substrate of wheat and barley bran by using a bacterial strain. The present study indicates that the extracellular α-amylase production through mix substrate of wheat and barley bran, this enzyme producing organism *B. amyloliquefaciens* MCCB0075 and substrate may have considerable potential for industrial application owing to its properties.

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