Growth Kinetics, Purification and Characterization of α-amylase Produced from *Bacillus licheniformis* DSM-1969 using Lignocellulosic Banana Waste as an Elicitor

Rashida Bashir,[a] Quratulain Syed,[a] Muhammad Waseem Mumtaz,[b] Farooq Anwar,[c,d] Nazamid Saari,[e]* and Ahmad Adnan [a,]*

In this study, banana waste was used to investigate its elicitation potential for induced production of α-amylase from *Bacillus licheniformis* DSM-1969. Initially, six different media were investigated to select the composition with optimal yield. A comparison of the fermentations in the stirred fermenter or shake flasks revealed that *B. licheniformis* DSM-1969 was more active to synthesize α-amylase in the fermenter as compared to the shake flask. In the shake flask during the exponential phase, the specific growth rate, generation time, and number of generations were 0.19 h^{-1}, 3.48 h^{-1}, and 5.16 h^{-1}, respectively, whereas in the stirred fermenter the above values were 0.3 h^{-1}, 2.31 h^{-1} and 5.21 h^{-1}, respectively. A significant difference was recorded in the specific substrate uptake rate and biomass growth yield during the exponential phase in the stirred fermenter in comparison to the shake flask. The enzyme was purified by ion-exchange chromatography using fast protein liquid chromatography (FPLC). α-amylase was purified 3.9 fold with a specific activity of 38.8 U/mg and molecular weight of 62 kDa. Characterization revealed that purified α-amylase remained stable over a broad pH and temperature range as compared to the crude enzyme. Activity of this novel extra thermo-stable α-amylase was stimulated to variable extents by Zn^{2+}, Co^{2+}, and Mn^{2+}, whereas EDTA and Hg^{2+} showed inhibitory effects.

Keywords: Green biotechnology; Banana waste; α-amylase; Elicitation; Purification; Characterization

Contact information: a: Department of Chemistry, Government College University Lahore, Lahore, Pakistan; b: Department of Chemistry, University of Gujrat, Gujrat, Pakistan; c: Department of Chemistry, University of Sargodha, Sargodha 40100, Pakistan; d: Department of Pharmaceutical Chemistry, College of Pharmacy, Salman bin Abdulaziz University, PO Box 173, Al-Kharj 11942, Saudi Arabia; e: Faculty of Food Science and Technology, University Putra Malaysia, UPM-43400 Serdang, Selangor, Malaysia;
** Corresponding authors: adnan.biochem.gcu@gmail.com, nazamid@upm.edu.my*

INTRODUCTION

In recent times, a wide spectrum of micro-organisms have been considered as potential sources of industrially important enzymes, where these enzymes can be used in a large number of industries, such as the food, feed, leather, textile, and paper industries (Singh *et al.* 2011). Among many other microbial strains, *Bacillus spp.* is one of the most efficient enzyme producers being studied for the production of starch-degrading enzymes like amylase from agro-industrial waste materials such as citrus peel, wheat straw, rice husk, banana waste, and bagasse (Sivaramakrishnan *et al.* 2006). Unfortunately, most of

the above-mentioned waste substrates are not being appropriately discarded in developing countries, leading to a major source of ecological pollution (Irshad *et al.* 2013). However, the range of cheap and readily available agricultural or agro-industrial residues represents an energy-rich source and can be used as microbial growth-eliciting substrates in fermentation processes. These residues are, in fact, one of the best reservoirs of fixed carbon in nature (Francis *et al.* 2003). Apart from this critical advantage in fermentation, such inexpensive substrates not only supply nutrients to the culture, but also serve as an anchorage for the microbial cells.

Recently, enzyme-based processing has received a great deal of interest among not only academics but also industrialists because of its technological and economic benefits in many areas of modern biotechnology (Asgher *et al.* 2014). Starch-degrading enzymes are a complex of three major enzymes that exhibit higher collective activity and degrade starch into glucose and other useful commodities. The α-amylases are extracellular enzymes that randomly cleave the 1,4-α-D-glycosidic bonds between adjacent glucose units in the linear amylose chain (Mohandas *et al.* 2010). β-amylases are exo-acting enzymes that cleave non-reducing ends of amylose, amylopectin, and glycogen molecules. Glucoamylase hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin (Singh *et al.* 2014).

Cost-effective production and availability are important considerations for successful exploitations of products at an industrial level. A potential method for low-cost fermentative production of enzymes involves the use of lignocellulosic waste materials because they contain carbohydrates that must be first converted into simple sugars and then fermented to obtain the product of interest. The estimated expenditure of the global market is 2.7 billion $US, which increased by 4% *per annum* until 2012. Different industries shared the above-mentioned estimated expenditure according to their requirement (*i.e.,* detergent shared 37%, textiles 12%, starch 11%, baking 8%, and animal feed 6%) out of the total 2.7 billion $US of the global market, while utilizing 75% of industrially produced enzymes (Das *et al.* 2011).

Bananas are one of the most consumed fruits in the world; the Sind province of Pakistan alone produces 350,000 tons of bananas per year. Each hectare of banana crop produces an abundance of banana wastes, which imposes a great environmental burden (Irshad *et al.* 2013). Keeping in view the importance of cheap and efficient production of amylases for industrial processes and the fact that the potential of amylases in different industries needs to be extensively explored, the present study was carried out to investigate the growth kinetics for improved yield using a laboratory-scale bio-reactor. The present study was also performed to purify and characterize the α-amylase from *B. licheniformis* DSM-1969 to present its potential for industrial purposes.

EXPERIMENTAL

Chemicals and Lignocellulosic Substrate

All the chemicals used were of analytical grade and purchased from Sigma-Aldrich (USA). Banana waste was obtained from a local fruit market, Lahore, Pakistan. Solid banana waste material was crushed into small pieces and washed three times (2

times per h) with warm water to minimize dust contamination. The washed substrate was then sun- and oven-dried (60 °C) and ground to fine 40-mm-mesh particle size and stored in air-tight plastic jars to avoid free moisture.

Pretreatment of Lignocellulosic Substrate

Moisture-free fine powdered substrate (20 g) was pretreated with 1% HCl in an Erlenmeyer flask (500 mL) at room temperature (30 °C) for 1 h, then autoclaved at 121 °C and 103 kPa for 15 min. Afterwards, the substrate slurry was filtered through four layers of muslin cloth, and both filtrate and residue were retained. Residues were washed four to five times with distilled water to remove extra acidity prior to being used for enzyme production and further analysis.

Microorganism and Inoculum Development

A pure culture of *B. licheniformis* DSM-1969 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH Inhoffenstr, Braunschweig, Germany. To develop a homogeneous inoculum suspension, a pure colony of *B. licheniformis* DSM-1969 was cultivated in an Erlenmeyer flask containing 30 mL of nutrient broth at 30 ± 1 °C for 48 h after sterilizing the broth at 103 kPa and 121 °C for 15 min. The inoculated bacterial culture was then incubated in a temperature-controlled shaking (120 rpm) incubator at 37 °C for 12 h for the development of a homogenous inoculum suspension.

Selection of Fermentation Medium

Initially, six different media were investigated for amylase production in 500-mL Erlenmeyer flasks to select the one giving the maximum yield. Further, to investigate the effect of banana waste as an elicitor, a control run for each medium was also executed by excluding the banana waste. The compositions of all six media were as follows:

Fermentation medium M-1 (g/L)
Banana waste, 3.0; Peptone, 5.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5

Fermentation medium M-2 (g/L)
Banana waste, 3.0; Soya peptone, 4.0, Meat extract, 3.0; KH_2PO_4, 1.0, $MgSO_4.7H_2O$, 0.3

Fermentation medium M-3 (g/L)
Banana waste, 3.0; $(NH_4)_3PO_4$, 5.0; Yeast extract, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; sodium citrate, 0.1; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.1; $MnSO_4.H_2O$, 0.1

Fermentation medium M-4 (g/L)
Banana waste, 3.0; Meat extract, 3.0; Soya peptone, 4.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5, $CaCl_2$, 2.0

Fermentation medium M-5 (g/L)

Banana waste, 3.0; $(NH_4)_2SO_4$, 5.0; K_2HPO_4, 1.0; KH_2PO_4, 3.0; Sodium citrate, 1.0; $CaCl_2.2H_2O$, 2.0; $FeCl_3.6H_2O$, 0.15; $FeSO_4.7H_2O$, 0.05; $MgSO_4.7H_2O$, 0.5; $MnSO_4.H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.001

Fermentation medium M-6 (g/L)
Banana waste, 3.0; Yeast extract, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 2.0

Fermentation in Stirred Fermenter

The fermentation medium with the highest yield (M-6) was selected from initial shake flask trials for further investigation using a laboratory-scale stirred fermenter. The fermentation was carried out in a 7.5-L bioreactor (New Brunswick C24KC, USA) with a 5-L working volume with the addition of 500 mL of freshly prepared culture inoculum. An antifoaming agent was added manually to control the foaming effect in the bioreactor. An aeration rate of 0.55 $L.L^{-1}.min^{-1}$ was kept constant during the fermentation, and the dissolved oxygen (DO) concentration was maintained at 20% saturation by controlling the stirrer speed between 1000 and 1200 rpm at 30 °C.

Determination of Biomass

The total cell concentration was determined by measuring the optical density at 600 nm using a UV-spectrophotometer. Dry cell weight was measured in triplicate. The tubes containing fermented broth were centrifuged at 10,000 g for 15 min at room temperature, and the supernatants were stored for further analysis. The pellets were re-suspended in 50 mM phosphate buffer at pH 7.0 and re-centrifuged at 10,000 g for 15 min in pre-weighed falcon tubes and dried at 80 °C until reaching constant weight.

Determination of α-amylase Activity

The α-amylase activity in the supernatants collected was determined at room temperature by the DNS method (Tanyildizi *et al.* 2005). Tubes containing fermented broth were centrifuged at 10,000 g for 15 min at room temperature, and the supernatant was collected carefully. The supernatant obtained was treated as crude enzyme, and the α-amylase activity was determined using the DNS method with glucose as a standard at 540 nm. Recorded activities are expressed as U/mL.

Determination of Protein Contents, Substrate, and CO_2

The protein contents of the crude and purified enzyme extracts were determined by the Bradford method (1976) using bovine serum albumin (BSA) as a standard. At the end of each experiment, the residual substrate was first saccharified by acid-catalyzed hydrolysis and then quantified by the DNS method (Idrees *et al.* 2013). The carbon dioxide produced during the fermentation was absorbed in NaOH by passing the exhaust air from the fermenter through a defined volume of 1.0 mL/100 mL solution of NaOH. Samples from the spent NaOH solution were titrated against a standard oxalic acid solution using phenolphthalein indicator. The amount of CO_2 was determined from the amount of NaOH consumed, according to the method described by Samun (1993).

Growth Kinetics and Carbon Mass Balance

To determine the specific growth rate (h^{-1}), the natural log of biomass (ln X) was plotted against time (t). The slope of the line gave the specific growth rate (μ) at any moment, which was then used to calculate the time required to double the biomass, *i.e.*,

$$\text{Generation Time (or doubling time } t_d) \text{ as } t_d = 0.693/\mu \quad (1)$$

The degree of multiplicity (n), *i.e.*, the number of generations produced in a particular time interval, was calculated using the equation given below:

$$n = 3.32 \log (X/X_0) \quad (2)$$

Biomass growth yield ($Y_{X/S}$) was determined as the ratio of the amount of biomass produced to the amount of the substrate consumed ($g_{Biomass}/g_{Substrate}$). A graph of changes in dry cell mass to changes in the amount of the carbon source consumed was plotted; the slope of the graph was $Y_{X/S}$. The substrate consumption rate (SCR) at any moment was determined as the slope of the graph between substrate consumption (gL^{-1}) and fermentation time and expressed in $gL^{-1}h^{-1}$. The specific substrate uptake rate (q_{sub}) was calculated as the ratio of substrate consumption rate (SCR) to total biomass.

Carbon Mass Balance Studies

Molar yield coefficient/carbon yield coefficient of biomass from the substrate ($Y^C_{X/S}$) was calculated as the ratio of moles of carbon in the form of biomass to the moles of carbon consumed as the substrate. The carbon yield coefficient of carbon dioxide from the substrate ($Y^C_{CO2/S}$) was calculated as the ratio of moles of carbon released in the form of carbon dioxide to the moles of carbon consumed as the substrate. The following formula was used for the mass balance calculations (Dauner *et al.* 2001).

$$C_{Substrate\ Consumed} = C_{Biomass\ Produced} + C_{CO2\ Produced} + C_{Extracellular\ Products} \quad (3)$$

Purification of α-amylase

The α-amylase was purified using ammonium sulfate precipitation and fast protein liquid chromatography (FPLC) using RESOURSE S (Amersham Biosciences, USA). Solid ammonium sulfate was added to the crude α-amylase to obtain 80% saturation at 0 °C with continuous stirring at 100 rpm. The precipitates were lyophilized followed by dissolution in phosphate buffer (pH 7.0) and dialyzed against 0.05 M sodium phosphate buffer for 24 h. The dialyzed sample was filtered through a 0.4-µm Millipore filter and loaded onto an anion exchange column RESOURCE-S (6.0 mL, 16 mm x 30 mm; Amersham Biosciences, USA) equilibrated with 50 mM sodium phosphate buffer with a flow rate of 1.0 mL/min. The protein was eluted with a linear gradient of NaCl (0 to 1 M) in the buffer. Both the enzyme activity and the protein contents were determined for each fraction, as described in the previous section.

SDS–PAGE for Determination of Molecular Weight

To determine the molecular weight of purified fractions of α-amylase, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a

5% stacking and 12% resolving gel using a Bio-Rad miniprotean II electrophoresis unit (Laemmli 1970). The protein marker ranged between 21 and 116 kDa (Sigma, USA).

Characterization of Purified α-amylase

Effect of pH and temperature on activity and stability

The free and purified forms were incubated at 25 °C for 15 min at varying pH (3 to 10) values. For the stability assay, amylase was incubated at 25 °C for up to 24 h without substrate before assaying. To investigate the effect of temperature, both forms were incubated at varying temperatures (20 to 80 °C) before conducting the enzyme assay. For stability studies, the enzymes were incubated at varying temperatures for 24 h in the absence of substrate. Residual enzyme activities were determined every 8 h.

Effect of activators/inhibitors

The effect of various organic (EDTA) and inorganic ions (Hg^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+}) as possible stimulators or inhibitors on purified amylase produced from was studied. The enzyme activities in each case were determined under the standard assay conditions described earlier.

Statistical Analysis

All experiments and enzyme assays were performed in triplicate; statistically evaluated results are presented as mean ± S.E. (standard error). The S.E values are displayed as Y-error bars in figures.

RESULTS AND DISCUSSION

Selection of Fermentation Medium

Different fermentation media were investigated for amylase production by *B. licheniformis* DSM-1969 in shake flasks. The medium M-6 was found to be the best, with 27 U/mL α-amylase and 16.1g/mL biomass produced. The activity profile (Fig. 1A) obtained from the medium with banana substrate revealed higher yields in terms of amylase activity than the media without substrate (Fig. 1B). This clearly suggests the elicitation role of the substrate in the fermentation process. The time taken by the culture organism for enzyme synthesis is dependent on the length of the lag phase and primary metabolism, which varies with the composition for different agricultural residues. It has been reported in the literature that low-cost substrates are suitably effective for microbial growth and enzyme production (Sen *et al.* 2009; Iqbal *et al.* 2011).

Fermentation Pattern in Shake Flasks and Stirred Fermenter

Figure 2 shows changes in the concentration of the carbon source, dry cell mass, and α-amylase activity as a function of fermentation time in shake flasks or stirred fermenter. It is evident from Fig. 2 that in both the shake flasks and the stirred fermenter the substrate was consumed and biomass was produced at a significant rate only in the first 24 h of fermentation. In both cases, the amylase continued to be released into the medium at a significant rate until hour 48 of fermentation; afterwards, a decreasing trend

was recorded. In the stirred fermenter, the fermentation rate was much faster than that in shake flasks, as indicated by carbon source consumption and biomass formation rates. This may be related to improved aeration and mass transfer in the stirred fermenter.

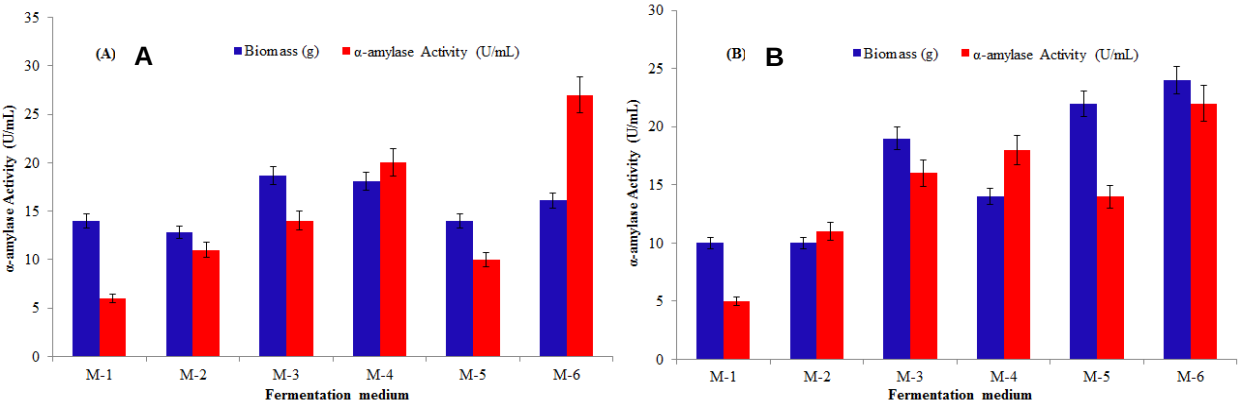

Fig. 1. Selection of a fermentation medium for induced production of α-amylase: (A) in the presence of lignocellulosic banana waste as an elicitor and (B) in the absence of banana waste

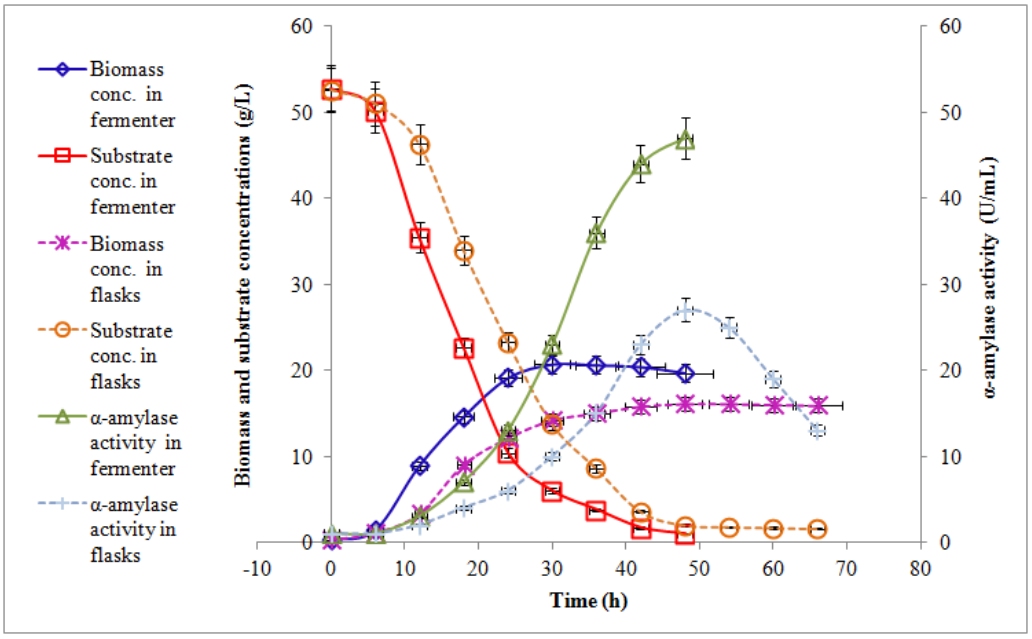

Fig. 2. Fermentation profile of *B. licheniformis* DSM-1969 α-amylase produced under optimum fermentation conditions using a 7.5-L glass bio-reactor and shake flasks

Growth Kinetics

Specific growth rates

The natural log of biomass (ln X) was plotted against time for both the shake flasks and the stirred fermenter (Fig. 3 A). In both cases, there was no lag in the growth, most probably due to the active nature of the juvenile vegetative inocula. In the stirred fermenter, during the first 12 h, the specific growth rate was 0.30 h^{-1}, and it gradually decreased to 0.11 h^{-1} in the next 6 h. This indicates that the true logarithmic growth phase lasted only for the first 12 h; however, in shake flasks, logarithmic growth was observed

for the first 18 h, during which the specific growth rate remained at 0.19 h^{-1}; afterwards, it gradually decreased, showing termination of the exponential growth phase. The generation times during the exponential phases were calculated to be 2.31 h and 3.48 h in the stirred fermenter and shake flasks, respectively. The number of generations during the 12-h logarithmic phase in the stirred fermenter was found to be 5.21, whereas the number of generations during the 18-h exponential phase in shake flasks was 5.16. After 48 h of process time, cell death dominated, which directly corresponds to exhaustion of nutrients and accumulation of toxins.

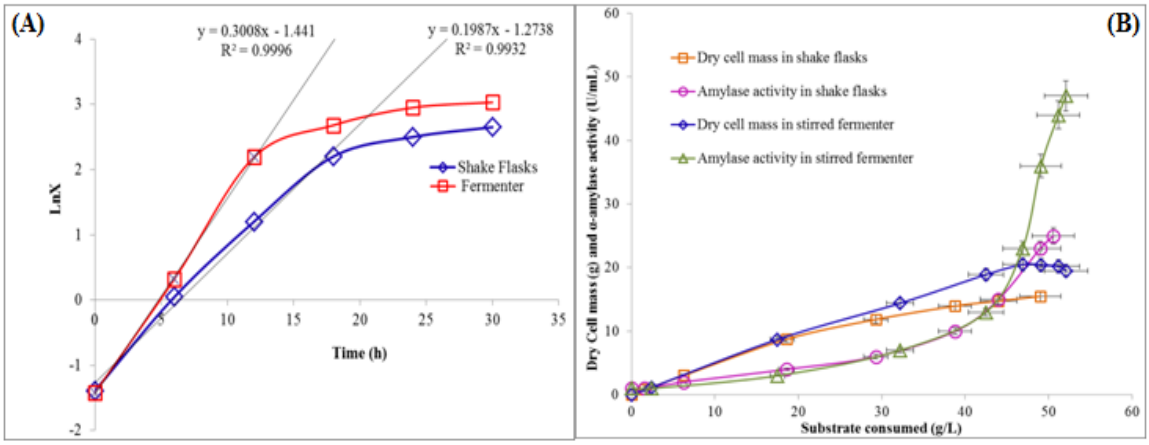

Fig. 3. (A) Specific growth rates of *B. licheniformis* DSM-1969 incubated for various fermentation times and (B) Biomass growth & α-amylase yield produced from *B. licheniformis* DSM-1969 under optimum fermentation conditions using a 7.5-L glass bio-reactor and shake flasks

Substrate consumption, mass balance, and specific substrate uptake rate (q_{sub})

Table 1 shows the concentration *vs.* time investigation for carbon source consumption, both in shake flasks and the stirred fermenter. It is evident that the rate of substrate consumption increased with time as the biomass increased and reached its maximum value of 2.52 g/L/h after 12 h of incubation in the stirred fermenter and 2.05 g/L/h after 18 h in shake flasks. The data represented in Table 1 show that during the 18-h exponential growth phase in the fermenter, about 55% of the carbon of the consumed substrate was incorporated into the biomass and around 44% was oxidized to CO_2 to provide maintenance energy. After 24 h, however, the metabolic activities slowed down and the major use of the substrate was to provide maintenance energy through its oxidation to CO_2.

A more important and informative parameter compared to substrate consumption rate is specific substrate uptake rate (q_{sub}). The value of q_{sub} remained relatively uniform (0.28 g substrate/g biomass/h) during the 12-h log phase in the stirred fermenter and (0.23 g substrate/g biomass/h) during the 18-h log phase in shake flasks. Uniformly high specific uptake rates during the exponential growth phase are related to the high activity of cells. In light of the results discussed above, it can be assumed that the substrate taken up by the biomass was used for three main purposes: i) producing new cell mass; ii) respiration to provide maintenance energy; and iii) to produce extracellular products. After 12 h of fermentation in the fermenter and 18 h in the shake flasks, the reproductive activities in terms of specific growth rate decreased and the substrate uptake per unit

biomass dropped significantly, as the major metabolic activity in the cells was respiration, providing the maintenance energy, as indicated by the CO_2 evolution rate in

Time (h)	Substrate Conc. (g/L)	Substrate Consumption (g/L)	Moles of C in the substrate consumed	Total CO_2 evolved (g/L)	Moles of C in CO_2	Molar yield coefficient of CO_2($Y^C_{CO2/S}$)	Biomass produced (g)	Moles of Carbon converted to Biomass	Molar yield coefficient of biomass ($Y^C_{X/S}$)
0	52.8	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	50.5	2.3	0.076	1.4	0.0318	0.418	1.14	0.042	0.55
12	35.4	17.4	0.58	10.2	0.232	0.4	8.66	0.32	0.552
18	20.0	32.1	1.07	18.9	0.429	0.4	12.1	0.532	0.497
24	10.4	42.4	1.41	26.1	0.593	0.42	18.87	0.669	0.4744
30	6.0	46.8	1.56	28.7	0.652	0.438	20.46	0.76	0.487
36	3.8	49.0	1.63	31.2	0.709	0.435	20.37	0.758	0.465
42	1.7	51. 1	1.7	33.6	0.7636	0.45	20.23	0.753	0.443
48	1.0	51.8	1.72	34.7	0.7886	0.46	20.03	0.745	0.433

the fermenter.

Table 1. Kinetic Parameters for the Production of α-Amylase from *B. licheniformis* DSM-1969 under Optimum Fermentation Conditions

Growth Yield ($Y_{x/S}$) and Product Yield ($Y_{p/S}$)

Figure 3 (B) presents a relationship between the substrate consumption and the formation of biomass and α-amylase at different time points, including the gradient of the curves at any point, which gives the biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$). It can be deduced from the graph that in the stirred fermenter, the biomass yield remained at 0.498 g biomass/g substrate (the economic coefficient = 2.0 g substrate/g biomass) during the 12-h exponential phase. In the shake flasks, the biomass growth yield on the basis of the carbon source remained at approximately 0.47 during the 18-h exponential growth phase (the economic coefficient = 2.1 g substrate/g biomass) and then dropped gradually. At the end of fermentation in the stirred fermenter and shake flasks, the overall enzyme yield on a substrate basis ($Y_{P/S}$) was 902 units/g and 494 units/g, respectively. In an earlier study, Haq *et al.* (2003) reported that the kinetic values $Y_p = X$, $Y_p = S$ and Q_p indicated that the production of α-amylase improved in the new medium compared to the original medium. The values of $Y_{x/S}$ and $Y_{p/S}$ were slightly lower in M-6 as compared to the other media, but the biomass yield of the enzyme was substantial.

Purification and SDS-PAGE Analysis of α-amylase

The results obtained after each purification step are summarized in Table 2. The enzyme produced was purified to homogeneity by three purification steps. The ultimate purification recorded was 3.9 fold, with an overall yield and specific activity of 8.9% and 38.8 U/mg, respectively (Table 2). The purified active fraction obtained from the FPLC RESOURCE-S column was further purified to homogeneity and resolved on 5% stacking and 12% running gel. The α-amylase was found to be a homogenous monomeric protein, as seen by the single band corresponding to 62 kDa on SDS-PAGE, which is within the range of the amylase family (Fig. 4). In earlier study, Rao *et al.* (2002) reported

molecular weights of raw starch digesting α-amylases from *B. lichenifromis* as 58.274 and 58 kDa, respectively.

Table 2. Purification Summary of α-amylase Produced from *B. licheniformis* DSM-1969 under Optimum Fermentation Conditions

Purification Steps	Volume (mL)	Enzyme Activity (U)	Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
Crude extract	200	9600	960	10.0	1.0	100
$(NH_4)_2SO_4$ Precipitation	50	3300	190	17.4	1.74	34.4
Ion-exchange chromatography (RESOURCE-Q)	20	1780	88	20.2	2.0	18.5
FPLC (RESOURCE-S)	9	855	22	38.8	3.9	8.9

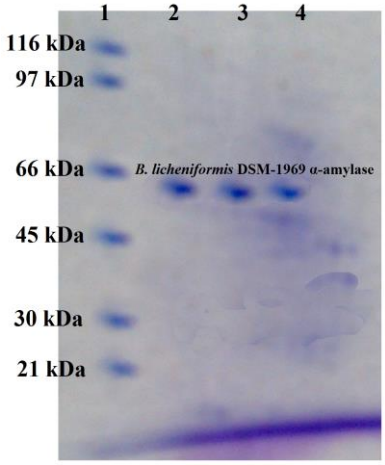

Fig. 4. SDS-Page analysis of purified α-amylase produced from *B. licheniformis* DSM-1969 Lane 1: Protein marker; Lanes 2, 3, and 4: Purified *B. licheniformis* DSM-1969 α-amylase

Characterization of α-amylase

Effects of pH and temperature

The pH-activity profile showed that free α-amylase was optimally active at the pH of 8, whereas the purified fraction showed measurable activity over a broad range of pH values, from 4 to 10. The enzyme activity decreased below and above the optimum pH range for the free counterpart of the α-amylase. The residual activity and stability profile showed that crude α-amylase was only 16% stable for 24 h at its optimal pH level, whereas purification at the level of homogeneity enhanced stability up to 65% and 98% for 24 h and 1 h, respectively, more than that of the unpurified enzyme (Fig. 5a). Lee *et al*. (2006) reported the highest activity of α-amylase from *Bacillus licheniformis* mutant in the pH range of 4.0 to 8.0. The purified α-amylase was optimally active at 50 °C; further increases in temperature decreased its activity. Crude α-amylase was only 9% active at 70 °C, while the purified fraction of the α-amylase showed better thermo-stability and retained 88% of its original activity at 70 °C after 24-h incubation (Fig. 5b).

For a variety of industrial applications, relatively high thermo-stability of an enzyme is an attractive and desirable characteristic (Asgher and Iqbal 2011).

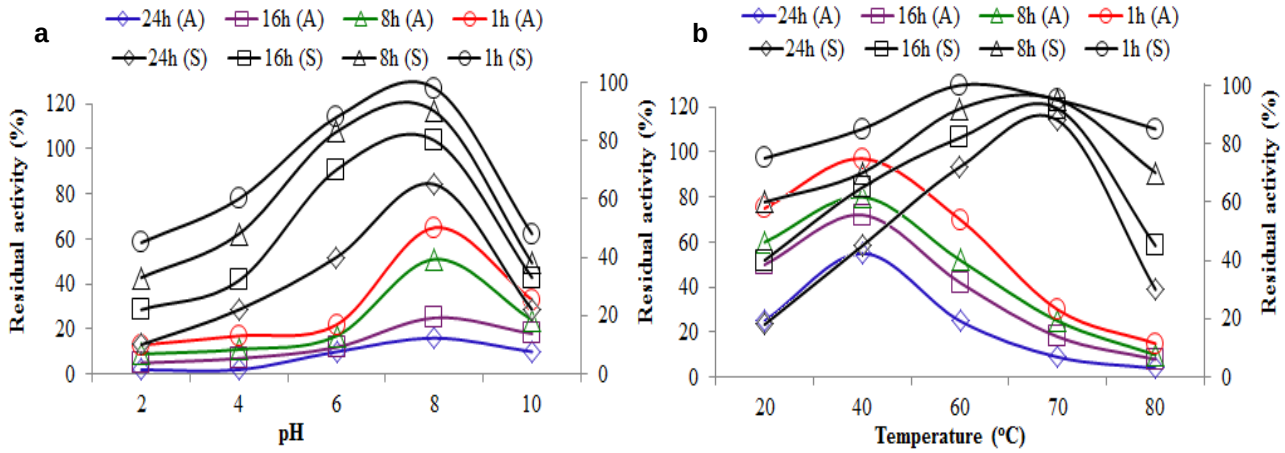

Fig. 5. Effect of pH (a) and temperature (b) on the activity (colored lines) and stability (black line) of *B. licheniformis* DSM-1969 α-amylase

Effects of various activators and inhibitors

A residual activity profile showed that the unpurified enzyme was completely inhibited by EDTA. Among the metal ions tested, only Hg^{2+} caused complete enzyme inhibition, whereas crude α-amylase retained 55, 72, and 65% of its activity in the presence of Zn^{2+}, Co^{2+}, and Mn^{2+} (Fig. 6). The purified fraction was 100% active in the presence of these metal activators. The purified α-amylase fraction was observed to present a noteworthy tolerance against inactivation by EDTA and Hg^{2+} (residual activity of purified enzyme was increased from 0 to 42% for EDTA and from 0 to 30% for Hg^{2+}) compared to the crude enzyme. EDTA is a metal chelating agent that forms a complex with inorganic groups of enzymes and therefore inhibits activity (Asgher and Iqbal 2011). Addition of Co^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, and Ni^{2+} into the enzyme production medium did not cause any alteration in the activity (Saha 2004). It has also been reported that the activator metal ions (Ca^{2+} and Na^{+}) protect enzymes against thermal denaturation and play a vital role in maintaining the active configuration of the enzyme at high temperatures (Asgher and Iqbal 2011).

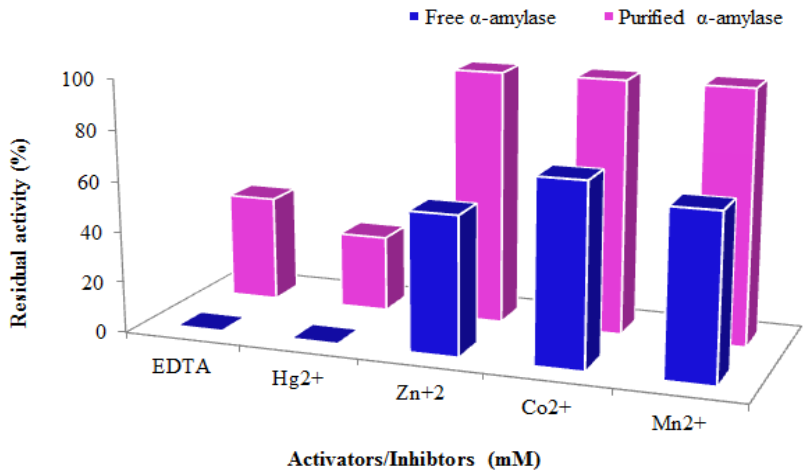

Fig. 6. Effect of different activators/inhibitors on crude and purified *B. licheniformis* DSM-1969 α-amylase

CONCLUSIONS

1. The results of this study showed the remarkable enzyme (α-amylase) production potential of bacterial strain of *B. licheniformis* DSM-1969 from agro–industrial residue banana waste.
2. *B. licheniformis* DSM-1969 α-amylase was purified 3.9 fold with a specific activity of 38.8 U/mg in comparison to crude extract.
3. The present α-amylase remained stable over a broad pH and temperature range which suggests its potential for various biotechnological applications.
4. Activity of this novel extra thermo-stable α-amylase was stimulated to a varying extent by Zn^{2+}, Co^{2+}, and Mn^{2+}, whereas EDTA and Hg^{2+} showed inhibitory effects.

ACKNOWLEDGMENTS

For financial support and laboratory facilities, the authors are grateful to the Department of Chemistry, University of Lahore, Pakistan.

REFERENCES CITED

Asgher, M., and Iqbal, H. M. N. (2011). "Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04," *BioResources* 6(4), 4302-4315.

Asgher, M., Shahid, M., Kamal, S., and Iqbal, H. M. N. (2014). "Recent trends and valorization of immobilization strategies and ligninolytic enzymes by industrial biotechnology," *Journal of Molecular Catalysis B: Enzymatic* 101, 56-66.

Das, S., Singh, S., Sharma, V., and Soni, M. L. (2011). "Biotechnological applications of industrially important amylase enzyme," *International Journal of Pharma and Bio Sciences* 2(1), 486-496.

Dauner, M., Storni, T., and Sauer, U. (2001). "*Bacillus subtilis* metabolism and energetics in carbon-limited and excess-carbon chemostat culture," *Journal of Bacteriology* 183(24), 7308-7317.

Francis, F., Sabu, A., Nampoothiri, K. M., Ramachandran, S., Ghosh, S., Szakacs, G., and Pandey, A. (2003). "Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*," *Biochemical Engineering Journal* 15(2), 107-115.

Haq, I., Ashraf, H., Iqbal, J., and Qadeer, M. A. (2003). "Production of alpha amylase by *Bacillus licheniformis* using an economical medium," *Bioresource Technology* 87(1), 57-61.

Idrees, M., Adnan, A., Malik, F., Qureshi, F. A., and Shahzad, C. (2013). "Enzymatic saccharification and lactic acid production from banana pseudo-stem through optimized pretreatment at lowest catalyst concentration," *EXCLI Journal* 12, 269-281.

Iqbal, H. M. N., Asgher, M., and Bhatti, H. N. (2011). "Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*," *BioResources* 6(2), 1273-1287.

Irshad, M., Anwar, Z., But, H. I., Afroz, A., Ikram, N., and Rashid, U. (2013). "The industrial applicability of purified cellulase complex indigenously produced by *Trichoderma viride* through solid-state bio-processing of agro-industrial and municipal paper wastes," *BioResources* 8(1), 145-157.

Laemmli, U. K. (1970). "Cleavage of structural proteins during assembly of head of bacteriophage T4," *Nature* 227(5259), 680-685.

Lee, S., Oneda, H., Minoda, M., Tanaka, A., and Inouye, K. (2006). "Comparison of starch hydrolysis activity and thermal stability of two *Bacillus licheniformis* alpha-amylases and insights into engineering alpha-amylase variants active under acidic conditions," *Journal of Biochemistry* 139(6), 997- 1005.

Mohandas, B. S., Prabhakar, A., Rao, R. R. K., and Madhu, G. M. (2010). "Optimization of process variables for amylase production using agro residual wastes by Taguchi methodology," *Chemical Product and Process Modeling* 5(1), 32.

Rao, M. D., Purnima, A., Ramesh, D. V., and Ayyanna, C. (2002). "Purification of α-amylase from *Bacillus licheniformis* by chromatofocusing and gel filtration chromatography," *World Journal of Microbiology and Biotechnology* 18(6), 547-550.

Saha, B. C. (2004). "Production purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*," *Process Biochemistry* 39(12), 1871-1876.

Samun, K. (1993). "Dissolved carbon dioxide measurement and its correlation with operating parameters in fermentation processes," *Biotechnology Progress* 9(6), 655-660.

Sen, S., Veeranki, V. D., and Mandal, B. (2009). "Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1," *Annals of Microbiology* 59(3), 531-538.

Singh, S., Dutt, D., and Tyagi, C. H. (2011). "Environmentally friendly totally chlorine free bleaching of wheat straw pulp using novel cellulase-poor xylanases of wild strains of *Coprinellus disseminatus*," *BioResources* 6(4), 3876-3882.

Singh, S., Singh, S., Bali, V., Sharma, L., and Mangla, J. (2014). "Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry," *BioMed Research International* 215748.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., and Pandey, A. (2006). "α-Amylases from microbial sources – An overview on recent developments," *Food Technology and Biotechnology* 44(2), 173-184.

Tanyildizi, M. S., Özer, D., and Elibol, M. (2005). "Optimization of α-amylase production by *Bacillus* sp. using response surface methodology," *Process Biochemistry* 40(7), 2291-2296.

Article submitted: April 17, 2014; Peer review completed: June 16, 2014; Revised version received and accepted: June 18, 2014; Published: September 24, 2014.