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Research Article

Production, Purification, and Characterization of Thermostable α-Amylase Produced by *Bacillus licheniformis* Isolate AI20

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An optimization strategy, based on statistical experimental design, is employed to enhance the production of thermostable α-amylase by a thermotolerant *B. licheniformis* AI20 isolate. Using one variant at time (OVAT) method, starch, yeast extract, and $CaCl_2$ were observed to influence the enzyme production significantly. Thereafter, the response surface methodology (RSM) was adopted to acquire the best process conditions among the selected variables, where a three-level Box-Behnken design was employed to create a polynomial quadratic model correlating the relationship between the three variables and α-amylase activity. The optimal combination of the major constituents of media for α-amylase production was 1.0% starch, 0.75% yeast extract, and 0.02% $CaCl_2$. The predicted optimum α-amylase activity was 384 U/mL/min, which is two folds more than the basal medium conditions. The produced α-amylase was purified through various chromatographic techniques. The estimated enzyme molecular mass was 55 kDa and the α-amylase had an optimal temperature and pH of 60–80°C and 6–7.5, respectively. Values of V_{max} and K_m for the purified enzyme were 454 mU/mg and 0.709 mg/mL. The α-amylase enzyme showed great stability against different solvents. Additionally, the enzyme activity was slightly inhibited by detergents, sodium dodecyl sulphate (SDS), or chelating agents such as EDTA and EGTA. On the other hand, great enzyme stability against different divalent metal ions was observed at 0.1 mM concentration, but 10 mM of Cu^{2+} or Zn^{2+} reduced the enzyme activity by 25 and 55%, respectively.

1. Introduction

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units [1]. Although α-amylases can be derived from several sources, including plants, animals, and microorganisms, microbial enzymes generally meet industrial demands. The major advantage of using microorganisms for the production of α-amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics [2]. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry [3]. Thermostable α-amylases are available from different sources and they have extensive commercial applications in starch processing, brewing and sugar production [4] designing in textile industries [5], and in detergent manufacturing processes [6, 7]. Each application of α-amylase requires unique properties with respect to specificity, stability, and temperature and pH values dependence [8]. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications [9, 10]. Thermophilic fermentation is also considered quite useful for technical and environmental purposes [11]. The advantages are, for

instance, a reduction in cooling costs, a better solubility of substrates, a lower viscosity allowing accelerated mixing and pumping, and reduced risk of microbial contamination. However, running α-amylase production processes at higher temperatures will require new process design and improved knowledge of thermophilic bacteria [4]. Enzyme overproduction can be achieved by both genetic manipulations and media engineering. As excretion of metabolism products is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy [12]. The classical method for medium optimization involves changing one independent variable keeping the other factors constant. This method is time-consuming and incapable of detecting the true optimum, due to the interactions among the factors [13] and this limitation of a single factor optimization process can be eliminated by different techniques. One of the techniques is response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process, is a collection of experimental strategies, mathematical methods and statistical inference [13, 14].

In the present study, we studied the optimization of three significant parameters affecting the production of thermostable α-amylase from *B. licheniformis* isolate and this was through implementation of response surface experimental design (RSM). After medium optimization the enzyme was applied to purification steps with the aid of various chromatographic techniques using an automated ÄKTA Prime Plus system. Mamo and Gessesse [15] have used ion-exchange chromatographic methods for the purification of two-starch-digesting thermostable α-amylase from thermophilic *Bacillus*. While, other investigators depend on gel filtration chromatographic technique, in purification of α-amylase enzyme from *Bacillus licheniformis* ATCC 9945a [16].

The outstanding properties of the pure enzyme were characterized and the kinetic parameters according to Lineweaver-Burk plot were calculated.

2. Experimental Section

2.1. Microorganism. The bacterial strain used in this work designated as AI20 was isolated from garden soil samples collected from Indonesia and selected among a group of thermophilic bacteria that has the capability of producing various enzymes with outstanding properties for different industrial applications.

2.2. Isolation of DNA from Bacterial Strain. An overnight culture of the target AI20 isolate grown at 50°C was used for the preparation of genomic DNA. The DNA was isolated using Promegakit according to the manufacturer's instructions.

2.3. Identification of Bacterial Strain. The bacterium was characterized and identified by 16S rRNA gene sequencing using universal primers [17]. The forward and reverse primers were of the following sequences, respectively: AGAGTTTGATCMTGGCTCAG and TACGGYACCTTGTTACGACTT. The PCR was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis [18] and the remnant mixture was purified using QIA quick PCR purification reagent (Qiagen). The 16S rRNA gene fragment (1442 bp length) was sequenced in both direction and Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit [19]. Also, the sequence has been deposited in the GenBank under accession no. HQ883968.

2.4. Enzyme Production Conditions. The amylase production was carried out in 250 mL conical flasks containing 50 mL medium with the following composition: 5 g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4, and 0.25 g/L $CaCl_2{\cdot}2H_2O$ incubated at 50°C under shaking conditions (200 rpm) and inoculated with 2.5% of 24 h old culture. After the specified cultivation time for each set of experimental trials, the culture broths were centrifuged at 10,000 g for 10 min and the cell free supernatant was used for enzyme determination.

2.5. Qualitative Determination of α-Amylase Enzyme Activity. Qualitative determination of α-amylase was carried out using well cut or cup assay with some modifications [20]. The agar plates were prepared amended with 1% of starch and 1.5% of agar for well cut assay. After agar solidification, around 10 mm diameter of well was cut out aseptically using cork borer. The well was filled with the culture filtrate (100 μL), incubated overnight at 50°C, overlaid with 1% of iodine solution, then the hydrolytic zone around the well (clear zone) is measured. The negative control is maintained by adding sterile water in a separate well.

2.6. Quantitative Determination of α-Amylase Enzyme Activity. Quantitative determination of α-amylase was carried out based on Fuwa's colorimetric method [21] of iodine-starch color reaction with slight modification, where 50 μL of diluted enzyme in 50 mM phosphate buffer pH 7 was mixed with 100 μL of 1.1% of the prewarmed soluble-starch in the same buffer and the reaction mixture was incubated at 50°C for 10 min. After incubation the reaction was stopped by adding 250 μL from stopping solution (0.5 N Acetic acid and 0.5 N HCl prepared with ratio 5 : 1) then 100 μL from the reaction mixed with 1 mL iodine reagent (0.01% I_2 dissolved in 0.1% KI). The reduction in color was measured at 660 nm against control which prepared by the same method except that the stopping solution added before addition of the enzyme. One unit of α-amylase activity was defined as the amount of enzyme decreased the absorbance of 660 nm by 0.1 in 10 min.

2.7. Determination of Total Carbohydrates Concentration. Total of carbohydrates was assayed according to anthrone method [22, 23] and sucrose was used as standard. One mL of cell free supernatant was diluted to 500 mL with distilled water. A one mL of diluted sample is mixed with 5 mL of

0.2% anthrone reagent (0.2 g anthrone reagent in 100 mL of concentrated H_2SO_4) then immersed immediately in ice for 5 min. After that the tube was heated in a boiling water bath 10 min. then kept at room temperature for 60 min. and the developed color was measured at 620 nm.

2.8. Optimization of α-Amylase Production

2.8.1. Preoptimization Experiment by One Variable at Time (OVAT) Approach. The production level of α-amylase was determined upon growing of the selected isolate on several α-amylase production media, namely, M1, M2, M3, M4, M5, and M6 as reported in the literatures [20, 24–28], respectively. The medium which gave the highest yield of α-amylase was selected as basal medium for subsequent medium formulation. One Variable At Time (OVAT) method was applied initially to test the effect of different pH values ranged 5–9, incubation temperature ranged 30–50°C and inoculum size ranged 1–10% on α-amylase activity. Also, calcium chloride and different carbon/nitrogen sources have been tested.

2.8.2. Growth Pattern in Formulated Basal Medium. The growth, α-amylase production, starch consumption, and pH change are monitored in the selected medium: 5 g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4, and 0.25 g/L $CaCl_2{\cdot}2H_2O$ under optimal conditions of 7, 45°C and 2.5% for pH, temperature and inoculum size, respectively.

2.8.3. Optimization of Thermostable α-Amylase Production by Response Surface Methodology (RSM) Box-Behnken Design. To identify the optimum point for the significant variables, a response surface methodology using Box-Behnken design [29] was adopted for improving extracellular α-amylase production. As presented in Table 1, the previous three factors were prescribed into three coded levels, coded −1, 0, and +1 for low, middle, and high concentrations (or values), respectively. Table 2 represents the design matrix of sixteen trials experiment. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and production of the α-amylase enzyme. The equation for the three factors [29] was:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 \\ & + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2, \end{aligned} \quad (1)$$

where Y is the predicted enzyme production, β_0 model constant; X_1, X_2, and X_3 independent variables; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are cross-product coefficients; β_{11}, β_{22}, and β_{33} are the quadratic coefficients. Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Experiments were performed in triplicate and mean values are given.

Table 1: The levels of variables chosen for the Box-Behnken optimization experiment.

Variables	Variable code	−1	0	+1
Starch	X_1 (g/L)	5.0	7.5	10.0
Yeast extract	X_2 (g/L)	7.5	10.0	12.5
$CaCl_2$	X_3 (g/L)	0.1	0.15	0.2

2.8.4. Statistical Analysis of Data. The data of enzyme activity were subjected to multiple linear regressions using Microsoft Excel 97 to estimate t-values, P values, and confidence levels which is an expression of the P-value in percent. The optimal value of enzyme activity was estimated using the *Solver* function Microsoft EXCEL tools.

2.9. Purification of α-Amylase Enzyme

2.9.1. Purification Using Chromatographic Techniques. The cell-free culture supernatant was dialyzed and concentrated using the Labscale TFF filtration system (Millipore, Bedford). The concentrated culture was then applied to an anion-exchange chromatographic (diethylaminoethyl, HiPrep 16/10 DEAE-Sepharose FF, Pharmacia, Sweden) column equilibrated with 20 mM Tris-HCl buffer pH 8. The bound enzyme was eluted at flow rate 1 mL/min by using a linear gradient from 0–100% of 1 M NaCl in 20 mM Tris-HCl buffer pH 8. Fractions containing α-amylase activity were pooled together and concentrated using the Labscale TFF filtration system (Millipore, Bedford). The concentrated enzyme was applied to gel filtration column (HiPrep 16/60 Sephacryl S-100 HR, Pharmacia, Sweden) preequilibrated with 50 mM phosphate buffer and 200 mM NaCl at pH 8, and eluted by using the same buffer at flow rate 0.8 mL/min. Fractions with high α-amylase enzymatic activity were collected and pooled together. The pooled fractions applied to dialysis procedure using the ultrafiltration tubes at a speed of 5,000 rpm (3,030 g) for 20 min at 4°C in Centricon 10 (Amicon, USA) ultrafiltration concentrators (membrane cut off of 10 kDa).

2.9.2. Protein Determination. Protein concentration was assayed by the method of Lowry [30]. Bovine serum albumin (Boehringer) was used as standard.

2.9.3. SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed on 12% running gel as described by Laemmli [31] and the resolved protein visualized by silver staining following standard procedure as described by Heukeshoven [32]. A low molecular range protein standard (Spectra Multicolor Broad Range Protein Ladder, Fermentas) was used as molecular mass marker.

2.9.4. Effect of Temperature and pH Value. To determine the optimum temperature for starch hydrolysis, the activity of the purified α-amylase enzyme was measured under standard pH 7 using phosphate buffer, after 10 min of reaction at different temperatures ranging from 20°C to 100°C. Similarly, to

Table 2: Box-Behnken factorial experimental design, representing the response of thermostable α-amylase enzyme activity as influenced by starch, yeast extract, and $CaCl_2$.

Trial no.	Starch X_1	Yeast extract X_2	$CaCl_2$ X_3	Amylase activity measured (U/mL/min)	Amylase activity predicted (U/mL/min)
1	−1	−1	0	290.7	287.0
2	1	−1	0	320.8	330.2
3	−1	1	0	183.8	174.4
4	1	1	0	226.0	229.6
5	−1	0	−1	242.9	248.6
6	1	0	−1	311.7	304.3
7	−1	0	1	296.8	304.2
8	1	0	1	352.6	347.0
9	0	−1	−1	343.1	341.1
10	0	1	−1	182.7	186.5
11	0	−1	1	346.0	342.2
12	0	1	1	281.8	283.7
13	0	0	0	216.5	220.3
14	0	0	0	210.3	220.3
15	0	0	0	230.7	220.3
16	0	0	0	223.8	220.3

determine the optimum pH value, the activity of the purified wild type α-amylase enzyme was measured after 10 min of reaction at different pH values within the range 3–10.6. Citrate, phosphate, Tris-HCl, and glycine-NaOH buffers were used for the following specified pH values: 3–6.2, 5.8–8, 7.6–8.9, and 8.6–10.6, respectively. The peak of the α-amylase activity was detected and considered as the optimal point. The thermal stability of α-amylase was determined by measuring the residual enzyme activity after incubating an aliquot of the enzyme at 50°C, 60°C, and 70–80°C for 24, 20, and 1.5 h, respectively. The enzyme was incubated at tested temperature without substrate then the residual activity was measured under optimal conditions of temperature and pH values.

The pH stability of α-amylase was determined by measuring the residual enzyme activity after incubating an aliquot of the enzyme at pH ranged from 4 to 12 for 24 h. The enzyme was incubated at tested pH and specified buffer without substrate, then the residual activity was measured under optimal conditions of temperature and exposed pH buffer.

2.9.5. Determination of Kinetic Parameters. Determination of the kinetic parameters for the hydrolysis of α-amylase enzyme were calculated according to the method of Lineweaver-Burk plot [33] by using the starch as substrate in concentrations ranged from 0.5 to 1.75%. Values of maximum rate; V_{max} (mU/mg/min) and Michaelis-Menten constant; K_m (mg/mL) were determined and all the reactions were carried out at 60°C and pH of 7.5.

2.9.6. Effect of Different Solvent, Detergents, Anionic Surfactant, Chelating Agents, and other Chemicals. The amylolytic activity was estimated in the presence of different solvents: ethanol, methanol isopropanol, and acetone at different concentrations 1, 10, and 20%, detergents: Triton X-100, Tween 20, and Tween 85 at concentrations 0.25, 0.5 and 1.0%.; surfactant: SDS at concentrations 0.25, 0.5, and 1.0%; chelating agent: EDTA at concentrations 0.01, 0.05, and 0.25%. Other chemicals such as EGTA and glycerol were tested at concentrations 0.01, 0.05, and 0.25% and 1, 10, and 20%, respectively.

2.9.7. Effect of Different Divalent Metal Ions. The effect of different metals, such as Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} on the activity of the purified wild type α-amylase enzyme was measured under optimal conditions of temperature and pH, after 10 min of reaction at different metal concentrations 0.1, 1.0, and 10 mM. The relative activity was measured based on the untreated enzyme with the tested metal.

3. Results and Discussion

3.1. Isolation, Characterization, and Molecular Identification of Thermostable α-Amylase Producing Bacteria. In a preliminary screening program for isolation of α-amylase-producing bacteria, 200 isolates were obtained from soil and water samples tested. Qualitative determination of α-amylase activity was performed using well cut assay, where the potent isolate coded AI20 was selected.

In order to identify the isolate on a molecular basis, the 16S rDNA gene was amplified, sequenced, and deposited into GeneBank with the accession number (ac: HQ883968). The sequence analysis revealed a close relation to *B. licheniformis* AIS72 (ac: GU967452), with the maximum identity (99%).

Table 3: Medium screening for thermostable α-amylase enzyme production by *Bacillus licheniformis* AI20.

Medium code	α-Amylase activity (U/mL/min)
M1	2.0
M2	61.2
M3	2.0
M4	157.4
M5	166.5
M6	2.0

3.2. Preoptimization Experiment by One Variable at Time Approach. The production of starch hydrolyzing enzyme by *B. licheniformis* AI20 was tested using different media supporting α-amylase production (Table 3). The highest yield (166.5 U/mL/min) achieved using the basal production medium with the following formulation: 5 g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4 and 0.25 g/L $CaCl_2{\cdot}2H_2O$. The screening for pH, temperature, inoculum size, different carbon/nitrogen sources, and $CaCl_2$ (data not shown) indicated that, the optimum level of these variables are pH 7, temperature 45°C, and inoculum size of 2.5%. It was found that the variables, namely, soluble starch, yeast extract, and $CaCl_2$are considered the most significant factors affecting enzyme production.

3.3. Growth Monitoring. The basal medium with a composition of 5 g/L soluble starch, 5 g/L yeast extract, 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 3 g/L KH_2PO_4 and 0.25 g/L $CaCl_2.2H_2O$, showed a typical growth pattern, where microbial growth is concomitant to starch consumption reaching maximum specific growth rate (μ = 0.31/h) and substrate consumption (Q_{starch} = 0.75 g/L/h) as shown in Figure 1. The enzyme production started after 12 h of growth and increased exponentially after 24 h to reach a maximum activity and hence steady production (177 U/mL/min) after 40 h with a maximum production rate ($Q_{Amylase}$ = 11.97 U/mL/h) after 26 h.

3.4. Response Surface Methodology for Optimization of α-Amylase Production. Response surface methodology is considered to be one of the widely applied methods for optimization the enzyme production [34–36]. In order to approach the optimum production region of the α-amylase activity, the significant independent variables (starch, yeast extract, and $CaCl_2$) were studied, each at three levels: −1, 0 and +1 as represented in Table 1. The 16 trials design matrix illustrating Box-Behnken design was represented along with the experimental results of α-amylase activity (Table 2). All trials were performed in triplicate and the average of the observations was used. The main results of this study are presented in Figure 2, which represents the expected α-amylase response and correlation between variables in three dimensional plots. In Figures 2(a) and 2(b), it is to be seen

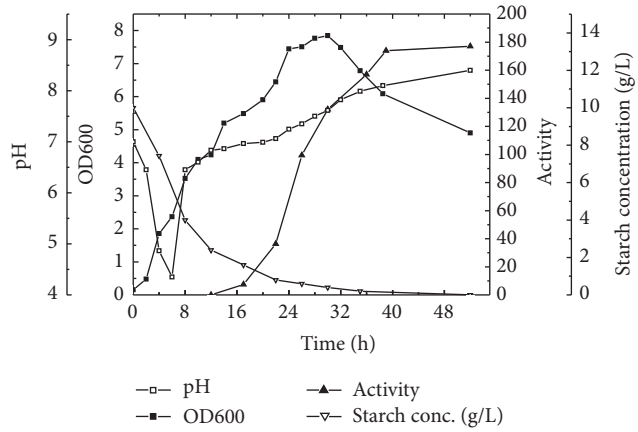


Figure 1: Time course of growth and α-amylase activity by *Bacillus licheniformis* AI20 isolate in basal medium.

that, the effects of pairs of factors were additive since there are low interactions between the starch-yeast extract and $CaCl_2$-starch pairs. Figure 2(c) showed nonadditive effects of yeast extract and $CaCl_2$ due to the significant interaction between them.

By additive of the two-factor effects, it is meant that the effect of one factor on the response does not depend on the level of the other factor. In Figure 2(a) it is obvious that maximum α-amylase activity was attained at lower levels of yeast extract (7.5 g/L) and higher levels of starch (10 g/L). Figure 2(b) illustrates that increasing $CaCl_2$ concentration in the medium independent on starch concentration led to increase in α-amylase activity. The optimum point deduced from Figure 2 is in accordance with the mathematically calculated optimum point. For α-amylase optimal production, within experimental constrains, a second-order polynomial function was fitted to the experimental results (nonlinear optimization algorithm):

$$\begin{aligned} Y = {} & 220.32 + 24.62X_1 - 53.29X_2 + 24.58X_3 \\ & + 3.00X_1X_2 - 3.24X_1X_3 + 24.04X_2X_3 \\ & + 23.8{X_1}^2 + 11.21{X_2}^2 + 56.88{X_3}^2, \end{aligned} \quad (2)$$

where X_1, X_2, and X_3 are the starch, yeast extract, and $CaCl_2$, respectively. At the model level, the correlation measures for the estimation of the regression equation are the multiple correlation coefficients R and the determination coefficient R^2. The closer the value of R is to 1; the better is the correlation between the measured and the predicted values. In this experiment, the value of R was 0.99 for activity of α-amylase. This value indicates a high degree of correlation between the experimental and the predicted values. The optimal level for α-amylase activity, being a measure of fit of the model, indicates that about 1.3% of the total variations are not explained by the enzyme activity. The optimal levels of the three components as obtained from the maximum point of the polynomial model were estimated using the Solver function of Microsoft Excel tools, and found to be: 10 g/L starch, 7.5 g/L yeast extract, and 0.02% $CaCl_2$ with

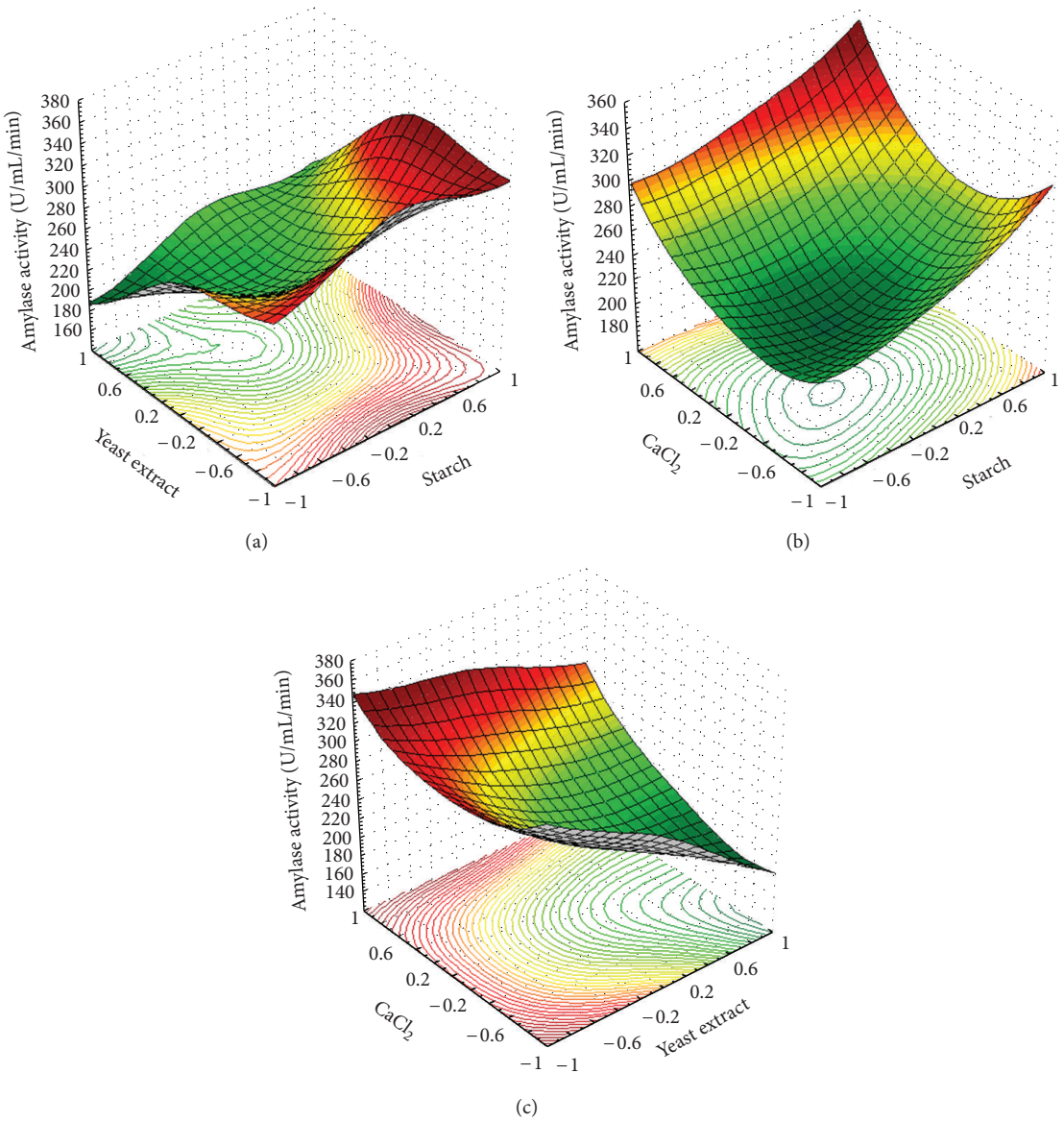


FIGURE 2: The response surface of α-amylase activity by *Bacillus licheniformis* AI20 as a function of (a), starch and yeast extract, (b), $CaCl_2$ and starch and (c), yeast extract and $CaCl_2$ in the culture environment.

predicted activity of 384.4 U/mL/min. The optimal value of the enzyme activity is more than two folds of the basal medium conditions. This reflects the necessity and value of optimization process. Results obtained in this study are in accordance with others findings where it reported that soluble starch, yeast extract, and $CaCl_2$ play an important role in enhancing the α-amylase activity [37]. The α-amylase enzyme is known to be a calcium metalloenzymes having at least one calcium ion associated with its molecules [38]. Also organic sources like yeast extract peptone usually have stimulating effect in α-amylase production [6, 39].

3.5. Verification of Model. In order to determine the accuracy of the quadratic polynomial, a verification experiment was carried out under basal and predicted optimal conditions monitoring growth, α-amylase activity, as well as total carbohydrate concentration in the optimized medium. The basal medium recorded a relatively low enzyme activity with a maximum of 177 U/mL/min attained after 40 h, where in optimized medium the maximum enzyme activity estimated was 375.5 U/mL/min after 96 h (data not showed). The predicted value from the polynomial model was 384.4 U/mL/min indicated high accuracy of the design about 97.8% and this high degree of accuracy is an evidence of the model validation under the following optimal conditions: 1% soluble starch, 0.75% yeast extract, 0.02% $CaCl_2$, 0.3% KH_2PO_4, and 0.02% $MgSO_4$ in pH 7 under cultivation temperature of 45°C and incubation time of 96 h.

3.6. Enzyme Purification. The steps that used for the α-amylase enzyme extraction and purification from *B. licheniformis* were showed in Table 4. It was indicated that during

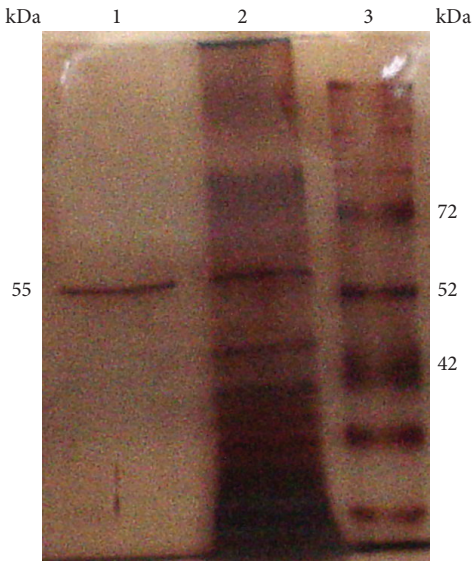


Figure 3: SDS Polyacrylamide gel electrophoresis (PAGE) showing the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20. Lane 1: The purified α-amylase enzyme after using gel filtration chromatography. Lane 2: The crude α-amylase enzyme in the culture filtrate. Lane 3: Low molecular range protein standard (Spectra Multicolor Ladder, Fermentas).

purification steps the total α-amylase activity reduced to about 554 U (about 12.6% of the whole α-amylase activity in the culture filtrate) in comparison with the initial α-amylase activity (about 4390 U), and also the total protein content reduced to 0.7 mg in comparison with the initial protein content of 347.8 mg. However, the specific activity at the end of the purification steps was found to be almost 749 U/mg comparing to 12.6 U/mg at the culture filtrate. It was also indicated that the α-amylase enzyme at the end of the extraction and purification steps was purified to 59.3-fold. Using ion-exchange chromatography such as the anion DEAE-Sepharose technique formed the main purification part and recovered about 46% of the total α-amylase enzyme with almost 8.4 fold of purification (Table 4). While, using the gel filtration technique as a last purification step in order to get a highly purified α-amylase enzyme which found to be only 12.6% of the original enzyme but with a 59.3-fold purification (Table 4).

3.7. Characterization of the Purified α-Amylase Enzyme

3.7.1. Molecular Mass of the Purified α-Amylase Enzyme. Determination of the purified α-amylase enzyme using SDS-PAGE electrophoresis with silver staining as described in Section 2 is considered not only for determining the molecular mass of the purified enzyme but also a method to indicate the enzyme purity. The molecular mass of the purified α-amylase enzyme was estimated to be about 55 kDa (Figure 3). Different molecular masses of the α-amylases from various *Bacillus* sp. including *B. licheniformis* ranging from 42 to 150 kDa [3] and from 23 to 50 kDa [40] have been reported.

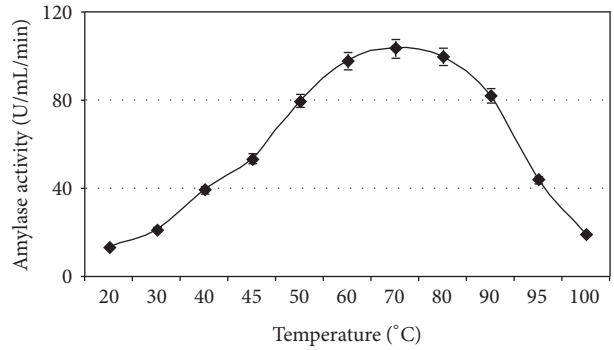


Figure 4: Temperature profile for the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20.

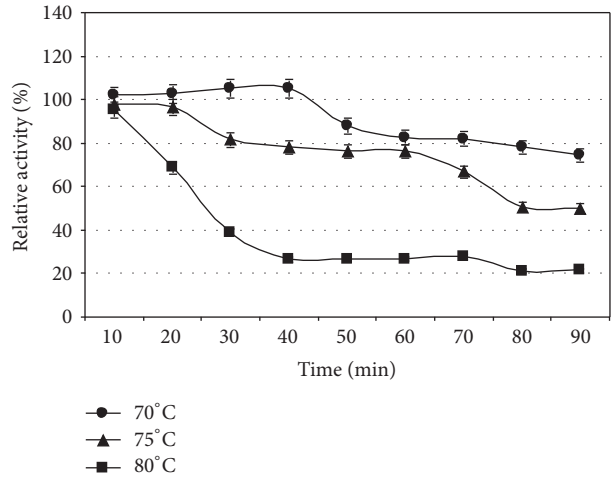


Figure 5: Studying the thermal stability for the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20.

3.7.2. Effect of Temperature on α-Amylase Enzyme Activity and Stability. For estimation of the optimum temperature of the purified α-amylase, the activity was determined at different temperatures from 20 to 100°C (Figure 4). The optimum temperature for enzyme activity was found to be ranged between 60–80°C (Figure 4). On the other hand, enzyme thermal stability was prevailed within 50–60°C for up to 12 h. Figure 5 showed that the purified enzyme retained 75, 50, and 20% of its activity at 70, 75, and 80°C for up to 90 min, respectively. Many other amylases from thermophile *Bacillus* showed to exhibited higher optimal temperature with more thermal stability than that obtained in our study [41, 42].

3.7.3. Effect of pH on α-Amylase Enzyme Activity and Stability. On pH profile, the purified enzyme had a preference to work over a wide range of pH (5.5–9.5), where an optimum pH

Table 4: Purification scheme of α-amylase enzyme extracted from *Bacillus licheniformis* AI20.

Purification step	Volume (mL)	α-amylase activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	148	4389.68	347.80	12.621	100	1
Concentrated culture	45	3055.95	196.65	15.540	69.617	1.231
DEAE Sepharose FF	27	2014.20	18.90	106.572	45.885	8.444
Sephacryl S-100 HR	5	554.25	0.74	748.986	12.626	59.344

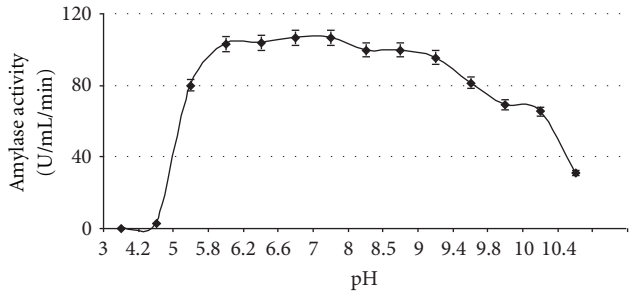


Figure 6: pH profile for the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20.

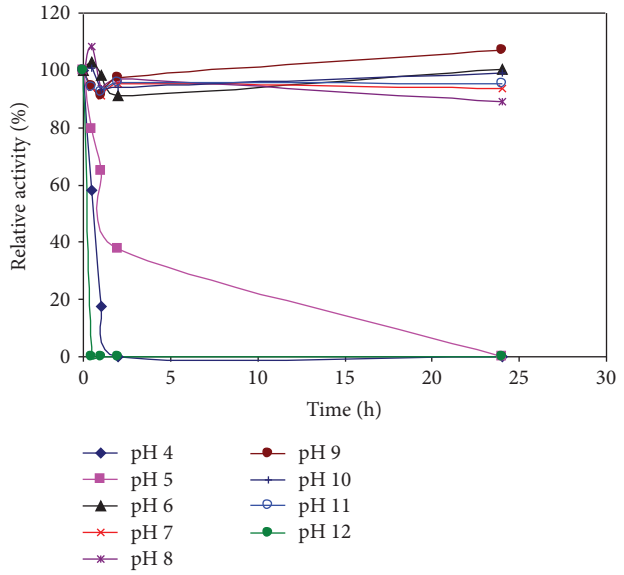


Figure 7: Studying the pH stability for the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20.

plateau was observed in range of pH from 6 to 7.5 (Figure 6). The enzyme retained 100% of its activity over a wide range of pH between 6 and 11 for 24 h, while exposed the enzyme to extreme pH value (pH 4 or 12) resulted in an inhibition in the activity within the first half an hour (Figure 7). Our results were highly similar with the literature results for optimal pH [3, 15, 16, 43] and for pH stability [15, 16, 43].

3.7.4. Effect of Substrate Concentration on α-Amylase Enzyme Activity. Studying the effect of substrate concentration on the α-amylase enzyme activity indicated a gradually increase

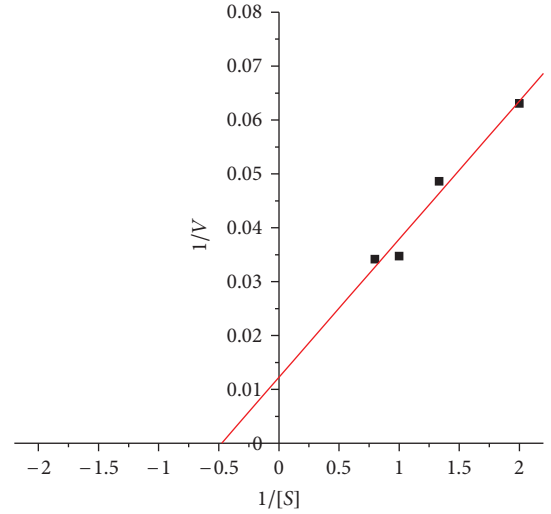


Figure 8: Lineweaver-Burk plot for the purified α-amylase enzyme produced by *Bacillus licheniformis* AI20.

in the activity by increasing of the substrate concentration up to 1.5%. Increasing the starch concentration by more than 1.25% resulted in an inhibition in the α-amylase activity. Most of the other α-amylases extracted from different regions also showed a substrate inhibition [26]. From the plotting according to Lineweaver-Burk, the maximum rate (V_{max}) was found to be 454 mU/mg and this result was matching with the result obtained on a highly immobilized thermostable α-amylase from *B. licheniformis* with a V_{max} value of 506 mU/mg [44]. The Michaelis-Menten constant (K_m) in our investigation was found to be 0.709 mg/mL (Figure 8). This result was more or less closed to other investigation on a thermostable and calcium-independent α-amylase of an extreme thermophile *B. thermoleovorans*, since K_m value was 0.83 mg/mL [26]. On the other hand, other investigators showed higher K_m value than our value such as the K_m value of 1.9 mg/mL on an alkaline chelator-resistant α-amylase from an alkaliphilic *Bacillus* sp. isolate L1711 [45] and the K_m value of 0.97 mg/mL obtained on α-amylase-produced by thermophilic *B. sphaericus* [46].

3.7.5. Effect of Different Solvents and Other Chemicals on α-Amylase Enzyme Activity. The purified α-amylase enzyme was tested against different solvents and other chemicals (Table 5). The enzyme showed a great stability against tested solvents up to 10% and increasing some solvents concentration to 20% (ethanol, methanol and isopropanol) showed slight inhibition in the enzyme activity. Studying different

Table 5: Effect of different solvents and other chemicals on the purified α-amylase enzyme activity produced by *Bacillus licheniformis* AI20.

Treatment	Residual α-amylase activity (%)		
	at 1%	at 10%	at 20%
Control	100	100	100
Ethanol	100	98.36	91.19
Methanol	96.44	95.34	93.24
Isopropanol	100	100	90
Acetone	100	97	97
Glycerol	100	100	99.47

concentrations of other chemicals such as glycerol showed no effect on the enzyme activity (Table 5).

3.7.6. Effect of Different Detergents, Anionic Surfactant, and Chelating Agents on α-Amylase Enzyme Activity. The effect of different detergents (Triton X-100, Tween 20, and Tween 85) on the α-amylase enzyme activity was studied and shown in Table 6. The obtained results show a good stability in the enzyme activity using different detergent up to 0.25% concentration, but only rising the Triton X-100 concentration up to 1% inhibited the enzyme activity by only 25% (Table 6). Our results are completely matched with the results obtained by other investigation on the α-amylase enzyme from *B. licheniformis* NH1 [43]. Since, 100% of the enzyme activity was recovered in the presence of 1% Triton X-100 and 1% Tween 20 after 1 h incubation at 40°C, while, rising the concentration of Triton X-100 to 5% caused moderate inhibition in the activity by less than 7% [43]. Slight inhibition of activity (35%) was observed in presence of anionic surfactant (SDS) at concentration 1.0%. This result matching with the results obtained by other investigators [41, 47] and the highly resistant for SDS could suggest that the enzyme has a potential in starch liquefaction and detergent industry. While, both tested chelating agents EDTA and EGTA caused an inhibition in the enzyme activity which found to be directly proportional with the increase in their concentrations (Table 6). The maximum inhibition 45% and 35% happened at concentration of 0.25% by EDTA and EGTA, respectively. Some investigators reported no effect of EDTA even at high concentrations [42, 48], while other investigators reported a slight loss in the α-amylase activity of 12% by using 10 mM EDTA [49].

3.7.7. Effect of Different Divalent Metal Ions on α-Amylase Enzyme Activity. The effects of various divalent metal ions at concentrations of 0.1, 1, and 10.0 mM on the α-amylase enzyme activity were assessed (Table 7). The enzyme showed a great stability against all tested metals (Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}) at 0.1 mM concentration. Increase the concentrations of Mg^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} cations up to 1 mM showed a slight decrease in the enzyme activity. In addition, increasing the concentrations of both Cu^{2+} and Zn^{2+} to 10 mM reduced the enzyme activity to 25 and 55%, respectively (Table 7). Most α-amylases from different sources are inhibited by metal cations such as Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} which is matching with our results [15, 50, 51].

Table 6: Effect of different detergents, anionic surfactant, and chelating agents on the purified α-amylase enzyme activity produced by *Bacillus licheniformis* AI20.

Treatment	Residual α-amylase activity (%)		
	at 0.25%	at 0.5%	at 1.0%
Control	100	100	100
Triton X-100	86.20	84.28	75.43
Tween 20	95.06	63.01	62.20
Tween 85	90.42	59.80	58.17
SDS	83.93	68.87	64.31
	Residual α-amylase activity (%)		
	at 0.01%	at 0.05%	at 0.25%
EDTA	74.17	64.31	56.75
EGTA	89.56	79.84	65.81

Table 7: Effect of different divalent metal ions on the purified α-amylase enzyme activity produced by *Bacillus licheniformis* AI20.

Treatment	Residual α-amylase activity (%)		
	at 0.1 mM	at 1.0 mM	at 10.0 mM
Control	100	100	100
Ca^{2+}	107.83	100.41	100
Mg^{2+}	95.44	92.52	104.96
Co^{2+}	102.61	91.28	92.09
Mn^{2+}	100.87	100.31	96.49
Cu^{2+}	99.67	93.55	24.73
Zn^{2+}	94.02	92.80	54.72
Hg^{2+}	42.07	0.00	0.00

However, completely inhibition in the α-amylase enzyme activity was observed by using Hg^{2+} at concentration up to 1 mM (Table 7) and these results are matching with different other investigators [15, 16, 52, 53].

The present study addressed the significant factors affecting α-amylase enzyme production by a newly isolated a thermotolerant *B. licheniformis* AI20 through the application of OVAT method. To attain the maximum yields of enzyme, the most effective factors were optimized further by applying RSM. The produced enzyme was purified and fully characterized, where it showed a high thermal, pH and solvent stability. In general, the studied properties of the purified native α-amylase from *B. licheniformis* AI20 make it a good candidate for wide applications; as additives in laundry detergents, stereospecific biocatalysts, and starch modification. These are excellent candidates in new industrial applications.

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