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Research article

Optimization of alpha-amylase production, immobilization and characterization by isolated *Bacillus lentus* under solid-state fermentation

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Key words: Alpha (α)-amylase, enzymes, immobilization, solid-state fermentation.

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Abstract

The agro-industrial by-product potato peels were utilized as a substrate for solid-state fermentation (SSF) using α-amylase producer *(Bacillus lentus),* isolated from Ain-Elseerah spring. Among twenty-eight isolates, six demonstrated clear zones of starch hydrolysis around the colonies when flooded with Gram's iodine solution. Maximum zone of hydrolysis was of 36 mm diameter, resulting in the production of 140 U/gm DS (dry substrate) α-amylase activity in 48 h. The optimization of α-amylase production was investigated under several affecting factors, including incubation-time; temperature; pH; and also the additional carbon and nitrogen sources. The highest α-amylase activity obtained (1980.18, 1980.42, 1730.18 ,1990.16 and 1560.24 U/gm DS) was with supplementation of 2% glucose, 1% yeast extract , After 72 h, at pH, 8 and temperature 40°C respectively. Seventy % of α-Amylase activity was retained after 1 h at 70°C and 60% activity after 1 h at 80°C. The enzyme was successfully immobilized in sodium alginate beads with immobilization yield of $7.5x10^2$ and $10.4x10^2$ for native and 50% enzyme respectively. Free and immobilized enzyme properties were elucidated.

Introduction

Enzymes are very important compounds because they function mainly as catalysts in biological and chemical processes [1]. Microorganisms are the most important sources for enzyme production, therefore, isolation and characterization of new promising strains using cheap carbon and nitrogen sources is a continuous process [2]. Thermophiles are the extraordinary microorganisms include a number of phyla with increasing potential in biotechnology [3]. These enzymes are of great significance in present day biotechnology due to their applications as food, baking, brewing, fermentation, bio-catalytic detergent applications, textile de-sizing, pulp and paper industries, also, analysis in medicinal and clinical chemistry [4]. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements, and elimination of neutralization steps, the microbial amylases meet industrial demands because, it is economical when produced in large quantities [2]. Alpha (α)-amylase can almost completely replace chemical hydrolysis of starch in the starch processing industry [5]. α-Amylases are endo-enzymes catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharide of various chain lengths [6]. Thermostable amylolytic enzymes are used for the hydrolysis and modification of starch to produce glucose [7]. It offers various advantages to industries i.e., the risk of contamination, the cost of external cooling, increase diffusion rate, resistant to denaturing agents and proteolytic enzymes [8]. Bioprocess method of amylase production is more effective than the other sources, since the technique is easy, cost-effective, fast, and enzymes of the required properties can be procured [9]. Mainly two types of amylases are produced by bacteria: Alpha-amylase and glucoamylase. α- amylase has found much commercial importance than glucoamylases, it degrades α-1-4 glucosidic linkage of starch [10]. Amylase isolated from bacteria has found more applications in food, textile, brewing, detergent and distilling industries. The amylase exhibited activity at a wide range of pH and temperature, desirable characteristics, which can lead to its application in detergents as additive and in textile de-sizing [1]; in pharmaceutical and sugar

industries [6]; alcoholic compounds production and paper industry [11]. Also, as baking; preparation of digestive aids; production of chocolate cakes; moist cakes; fruit juices and starch syrups that, paved a way for their large-scale commercial production [12]. α-Amylase requires unique properties with respect to specificity, stability, temperature and pH dependencies [13]. On the other hand, alkaliphiles are a group of extremophilic organisms that thrive at alkaline pH and many of their products, particularly enzymes, have found widespread applications in the industries particularly in detergent and laundry industries [7]. Alkaline Enzymes from alkaliphiles are stable in detergents due to their inherent tolerance to high pH [14]. α-Amylase has high catalytic efficiency and stability at alkaline pH ranging from 9 to 11 [15]. Alkaline α-amylase has the potential of hydrolyzing starch under alkaline pH and is useful in the starch and textile industries also, as an ingredient of detergents [16-17]. Several species of microorganisms including Bacillus species [18-19] have been isolated from various alkaline environments and those microorganisms produced large amounts of α-amylases. Thermostable alkaline amylases have extensive commercial applications with pH values higher than 8 and in starch saccharification, industries, as an ingredient in detergent for automatic dishwashers and laundries [13].

Immobilization technique is advantageous over free enzyme, since it increased catalytic activity and stability of enzyme [20]; easy recovery of enzyme; easy separation from product minimizing or eliminating protein contamination of product and repeated or continuous use of a single batch of enzyme [21].

Also, it does not detach from inert support matrix maximizing all benefits of immobilization [10] which, will ultimately save the enzyme, labor and overhead costs [6]. Immobilized enzymes are becoming increasingly popular as reusable; selective analytical chemical reagents in solid phase flow through reactors, as membranes in sensors and as films in dry reagents kits. Amylases play a vital role in biotechnological studies and rank an important position in the world enzyme market ranging from 25% to 33% [9].

Alginate the major structural polysaccharide of marine brown algae, contains β-D-mannopyranosyluronate and α-L-gulopyranosyluronate in regular (1-4)-linked sequences, whereas in the presence of divalent cations, especially $Ca^{[2+]}$, gelation occurs.

The aim of the present work was to screen various isolated α- amylase-producing bacterial strains. In like manner to identify (*Bacillus lentus* first time isolated from Ain-Elseerah spring, Cairo, Egypt) which had high level of α-amylase production among six isolated bacterial strains. Additionally to build up the optimization of solid state fermentation parameters (temperature, initial pH, incubation period, carbon and nitrogen sources) that enhances amylase production. Immobilization of the partial purified enzyme had been also discussed.

Experimental

Isolation and screening of various bacterial strains for α -amylase activity (Figure 1)

Water samples were collected from Ain-Elseerah spring in Cairo, Egypt. Bacteria were isolated by serial dilution and spread plate method using Luria Bertani agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, pH 7-7.5) [9]. The bacterial isolates were further sub-cultured to obtain pure cultures. Pure isolates were transferred to agar slants and kept at 4°C for further studies. Twenty-eight purified isolates were screened for α-amylase production on starch agar plates. For amylase detection after 48 hours of incubation at 30°C, 5 ml of 1% grams iodine solution (freshly prepared) was added to the culture plates. Six isolates showed zone of clearance around the colony representing degradation of starch, i.e. presence of amylase activity [22]. Hence, the strain, which exhibited maximum amylase activity, was selected for further study.

Characterization and identification of bacteria

The colony morphology of the potent α-amylase producer isolate was characterized based on Bergey's Manual of Determinative Bacteriology [23]. Investigated under the microscope with respect to colour, shape, size, and nature of the colony. Cultural and physiological characteristics also were studied [24].

DNA sequencing of the most potent bacterial strain was carried out with PCR amplicon. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Bio Systems, USA). Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system. Thereafter the phylogenic tree was prepared according to (nucleotide Blast) Search nucleotide databases using a nucleotide query.

Optimization of culture conditions

Selected strain *B. lentus* was subjected to various culture conditions to investigate the optimum culture conditions for α -amylase production, mainly incubation temperature (28, 37, 40, 45, 50, 55 & 60ºC), incubation time (24, 48, 72, 96 & 120 hours), pH (5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 & 10), carbon source (glucose, sucrose, maltose, lactose & starch), and nitrogen source (yeast extract, peptone, tryptone, and urea). Where the optimum values of pH, temperature and incubation period (8, 40°C and 72hrs respectively) were used for studying effect of carbon and nitrogen sources. All experiments were carried out, in duplicates, in 250ml Erlenmeyer flasks containing 5gm potato peels as fermentable substrate.

Solid-state fermentation for α -amylase production

Potato peels were washed and dried at 60°C for 24 h. Then, 5gm of crushed Potato peels was taken into a 250 ml Erlenmeyer flask and a salt solution containing (gm/l) (KH_2PO_4 2, NH_4NO_3 2, NaCl 1, and $MgSO_4.7H_2O$ 1); and

distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121ºC for 20 min. The experimental flasks were inoculated (2%) with freshly prepared inoculums, and incubated at the specific temperature. After incubation, 10 ml of distilled Water were added and the flasks were left on a rotary shaker for 1h. Then the fermented substrate was centrifuged at 3000 rpm for 20 minutes in a cooling centrifuge, and the supernatant was used for enzyme assay.

Enzyme assay

Alpha amylase activity was determined according to the method described by [25] with some slight modifications. Thus, 0.5 ml of 1% (w/v) soluble starch in 100 ml M phosphate buffer (pH 6.5) and 0.5 ml of enzyme extract, were added into a test tube and incubated for 30 min at 40°C. Thereafter, 1 ml of DNS (dinitrosalicylic acid) reagent was added to the mixture to stop the reaction. The mixtures were heated for 5 minutes in a boiling water bath. Before cooling, 0.3 ml of 40% Rochelle salt (Potassium sodium tartrate tetrahydrate) was added. After cooling to room temperature, the colour absorbance was measured at a wavelength of 575 nm using a UV-Visible spectrophotometer. The amount of reducing sugars was calculated using a standard curve prepared using glucose. The blank was prepared using 0.5 ml of enzyme extract that has already been boiled for 15 min. (to inactivate the enzyme) and all other assay conditions were followed. One enzyme unit was defined as the amount of enzyme that liberates 1 ml mole of glucose under the specified reaction conditions.

Enzyme Immobilization

Entrapment of enzyme in calcium alginate beads was studied. Thus, crude enzyme preparation was mixed with 2% sodium alginate prepared in 0.1M sodium phosphate buffer (pH 7.0) in two concentrations 5.0 and 2.5 mg protein/ ml. This mixture was dropped into 0.05M calcium chloride with stirring at room temperature. The obtained beads were stored for 20 h at 4ºC to allow complete gelation before use [26].

Determination of immobilization yield (Y_{imm})

It was calculated as the ratio of total activity yield of the immobilized preparation to the total activity of the soluble enzyme taken for immobilization [27].

Results and Discussion

Results

Screening of various bacterial isolates for α -amylase activity

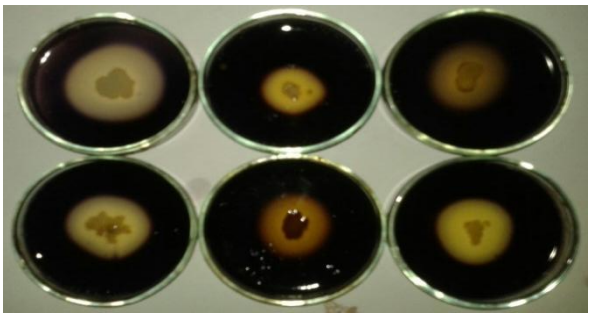

Figure 1. Exhibited different starch degradation zone of a six bacterial isolates

Identification of the most active bacterial isolate

The strain was found to be gram positive, catalase positive, motile, endospore positive and glucose positive. Then, it was identified as *B.lentus* (Figure 2) on the basis of sequence analysis of 16S rDNA.

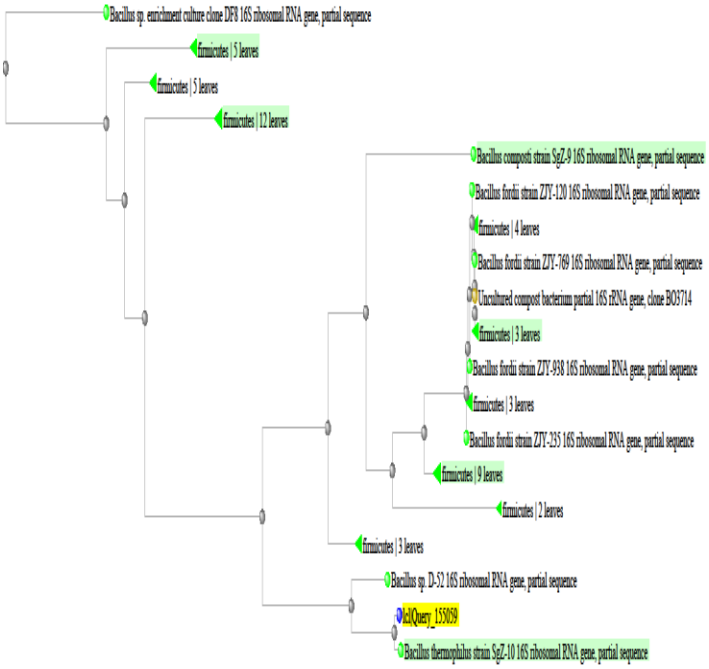

Figure 2. Phylogenetic tree based on 16S rDNA gene sequencing, showing the phylogenetic relationship of *Bacillus lentus* within representative species of the genus *Bacillus.*

Effect of Initial pH

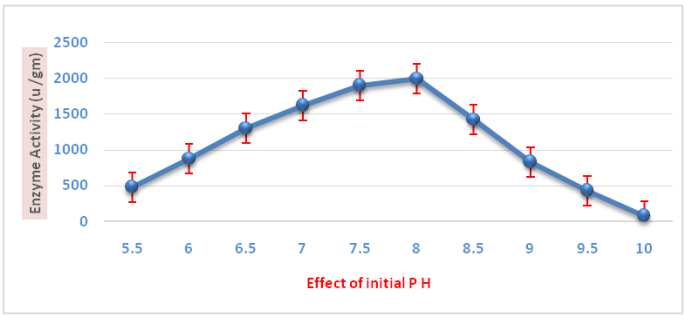

Figure 3. Effect of initial pH on amylase production by *Bacillus lentus* under SSF.

Concerning the initial pH optimization, Figure 3 showed that the production of α- amylase was optimized at pH 7.5 & 8. Where gradually increased from pH 6 to 8 and highest activity of (1990.***019*** U/gm) was observed at pH 8 while there was a decrease in enzyme activity above pH 8.5.

Effect of incubation time

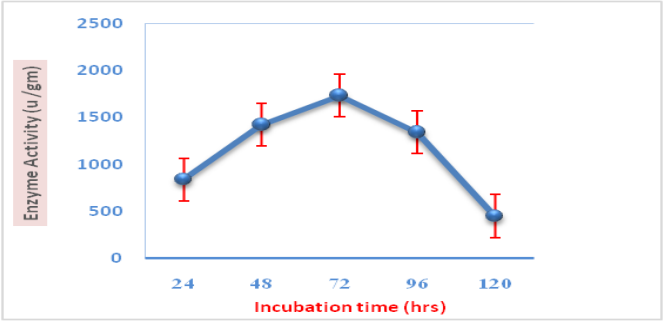

Figure 4. Effect of incubation time on amylase production by *Bacillus lentus* under SSF.

The time course for the production of α-amylase by *B. lentus* under SSF process using potato peels as the fermented substrate is depicted in Figure 4, after 24 h of incubation, 840.18 U/gm of the enzyme was produced, which increased to 1420.12 U/gm at 48 h. Then, followed by a slight decline in the enzyme production (1340.14 U/gm) at 96 h. the production reached the minimum level after 120 h.

Effect of incubation temperature

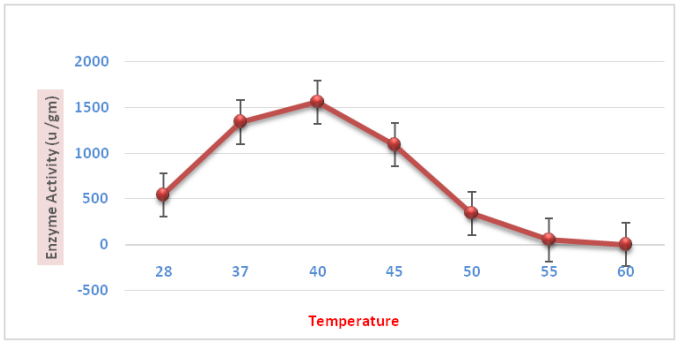

Figure 5. Effect of incubation temperature_on amylase production by *Bacillus lentus* under SSF.

Alpha Amylase enzyme production from potato peels by *B. lentus* showed an increase in activity (1560.12 U/gm) up to a temperature of 40°C. Thereafter, the enzyme showed a gradual reduction in its activity as the temperature was increased, (Figure 5). While, the enzyme activity dropped considerably after 50°C.

Effect of additional carbon sources

Figure 6 denotes the production of α -amylase on various additional carbon sources during the fermentation process. The supplementation of potato peels at 2% (w/v) concentration with Glucose and Lactose as sole carbon source, increase the enzyme activity to 1980.10 and 1340.15 U/gm, respectively. In addition, supplementation of starch in the medium resulted in marginally increased activities (1720.14 U/gm). On another hand, supplementation of potato peels with maltose has a little induction on the excretion of α-amylase (720.11 U/gm).

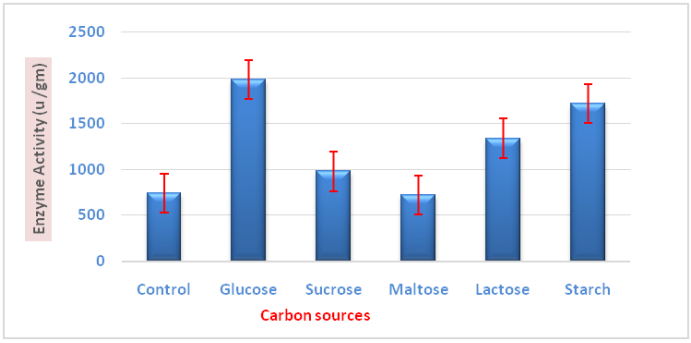

Figure 6. Effect of additional carbon sources on amylase production by *Bacillus lentus* under SSF.

Effect of Additional Nitrogen Sources

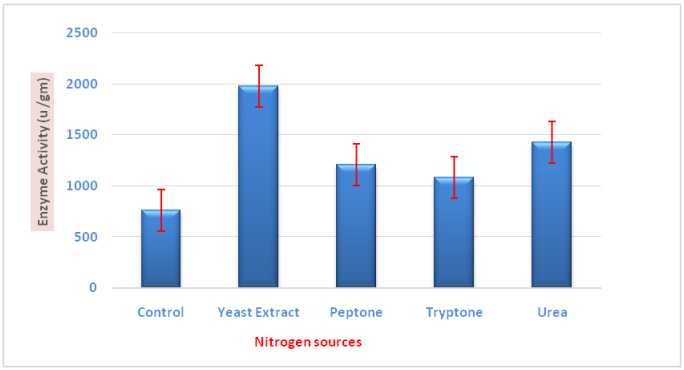

Figure 7. Effect of nitrogen sources_on amylase production by *Bacillus lentus* under SSF.

Supplementation of peptone and tryptone showed a good increase in the enzyme activity (1210.10 and 1080.12U/gm) respectively (Figure 7). While yeast extract resulted in the highest production of α-amylase, (1980.16U/gm) about 30% higher than urea 1430.12U/gm.

Enzyme Immobilization

(a): Native enzyme (b): 50% enzyme conc.

Figure 8. Alginate beads immobilization of amylase

The enzyme was successfully immobilized by entrapping in alginate through two enzyme concentrations.

Immobilization yield reached $7.5x10^2$ and $10.4x10^2$ for the native and 50% enzyme respectively (figure 8).

Characterization of immobilized enzyme

The obtained results (Figure 9) revealed that the optimum pH value of the free enzyme was 7.0 while it was 6.0 for the immobilized enzyme preparation. The 50% enzyme concentration reacts optimally at the pH value of 5.0.

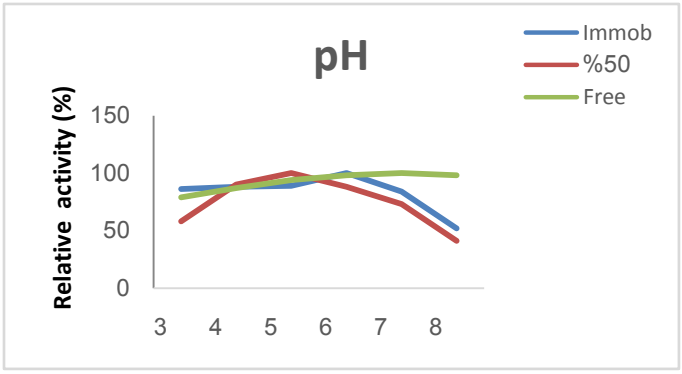

Figure 9. pH profile of free and immobilized α- amylase enzyme.

Temperature profile

Immobilized α- amylase exhibited its optimum activity at 60° C while 50% enzyme reacts optimally at 50° C. At the same conditions, the free enzyme preparation yielded its highest activity at 60° C (Figure 9).

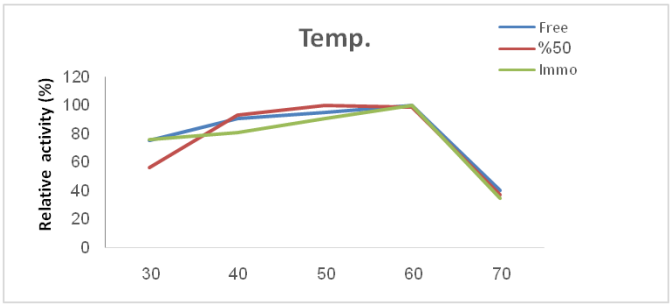

Figure 10. Optimum reaction temperature of free and immobilized enzyme.

Thermal stability

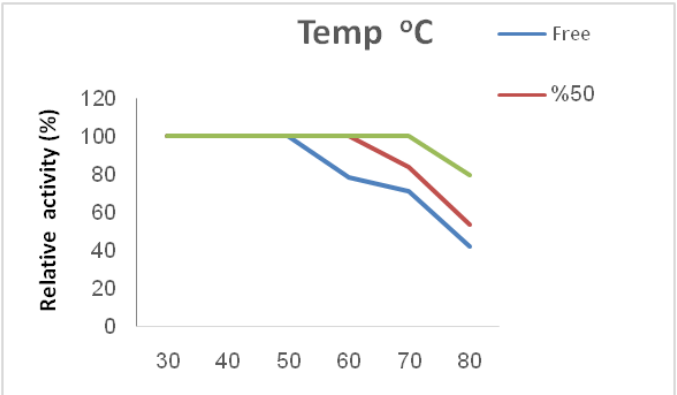

Figure 11. Thermal stability of free and immobilized enzyme.

Results showed that the immobilized enzyme had high thermal stability reaching to 70°C remaining 100% of catalytic activity. 50% enzyme concentration retained its thermal stability up to 60°C compared to 50°C to the free enzyme preparation (Figure 11).

Discussion:

The composition and concentration of media incredibly influence amylase production. The production of α- amylase was optimized at pH 7.5 & 8 (Figure 3). This result was in agreement with [3-28] who found that the highest amylase activity was at pH ranged of 7.5-8. In like manner it was reported that the optimum pH for amylase production by other species was exhibited at pH 7 [29], while other bacterial species exhibited optimal pH at 6.5-8.0 [30]. On other hand there was a decrease in enzyme activity above pH 8.5 (Figure 3); that is due to, the change of the initial pH of the medium leads to a change of the nature of the cell membrane that, affecting the growth of bacteria and the α-amylase production.

The enzyme assays exhibited a maximum α- amylase production after 72 h. (Figure 4) this result is in accordance of [31]. In like manner other studies suggested that the highest α- amylase production was after 2–4 days of incubation time [32]. Where the bacteria were in its exponential phase. It was followed by a slight decline in the enzyme production at 96 h. reached minimum level after 120 h. which indicated that the bacteria were in its stationary phase, due to nutrients depletion, and could have started producing secondary metabolites resulting in a lower yield of the enzyme [33]. Also, it may be due to the denaturation of the enzyme caused by the interaction with other components in the medium.

Whereas, the optimum temperature for α-amylase production by the isolate was 40°C (Figure 5). Similar findings were reported by Ashwini *et al.,* [31]. Further increase in temperature resulted in a decrease in α-amylase production, that may be due to enzyme denaturation which led to inhibit its activity [34]. In addition it may be due to the production of a large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation [35]. Temperature above 45°C results in moisture loss of the substrate, which affects metabolic activities and results in reduced α-amylase production [36]. On the other hand, several studies reported that most Bacillus sp. produced α-amylase at a temperature range from 37 till 60°C [37]. However, the optimum temperature for amylase production was at 50°C [38]; at 60°C [39] and at 65°C [40].

Results exhibited that the isolate showed great development on every one of the carbohydrates tested as sole carbon source (Figure 6). Where, glucose-grown organism exhibited the highest enzyme activity. It could be suggested that the enzyme production was growth-associated and the presence of simple sugars such as glucose in the medium has higher accessibility and suitability for the bacteria to utilize

and subsequently to produce a larger amount of enzyme [12]. In addition, supplementation of starch in the medium resulted in marginally increased activities. On another hand, supplementation of potato peels with maltose has a little induction on the excretion of α-amylase, in like manner it was found that addition of maltose exhibited the most reduced yield of α-amylase [41]. Also, sucrose exhibited low production of α-amylase that results could be suggested that maltose and sucrose are complex sugars which relatively more difficult to be utilized and required a longer time to be decomposed into simpler sugar [12].

Yeast, urea, peptone, and tryptone (Figure 7) enhanced the production of α-amylase. Supplementation of peptone and tryptone showed a good increase in the enzyme activity. On the other hand, yeast and urea were the best nitrogen sources for α-amylase production. Similar results were recorded by other researchers [42].While yeast extract resulted in the highest production of α-amylase. This was due to that yeast extract contains high nutritional amino acids, for instance, glutamic acid that was found to be significant for the cellular metabolism and could provide sufficient energy for the better bacterial growth which induced greater amount of α-amylase enzyme production [43].

Alginate beads immobilization of amylase

The enzyme was successfully immobilized by entrapping in alginate through two enzyme concentrations. The results of this study were in agreement with Mahajan *et al*., [44] who concluded and abstracted that sodium alginate of 1% was found to be best with respect to immobilization efficiency and diffusion of substrate into the beads. Moreover, this gel can withstand breakage. Concentration reacts optimally at the pH value of 5.0.

Characterization of immobilized enzyme

This pH shift to an acidic range is a phenomenon often observed when the enzyme is immobilized in an inert matrix. In contrast to our results, Torabizadeh *et al.,* [45], reported that optimum pH for free and immobilized were found to be 5.5 as no significant changes in the pH optimum of the enzyme after cross-linked enzyme aggregates. In addition, Homaei & Saberi [46] abstracted that optimum pH of α- amylase immobilized on gold nanorods was shifted from 6 to 7 upon immobilization.

Temperature profile

According to Figure 10 optimum immobilized α- amylase activities were in the same line with Swarnalatha., *et al*., [47] who stated that the optimum temperature for maximum enzyme activity for the immobilized and free α- amylase are identified to be 40^{o}C. One of the most advantages of enzymes immobilization was increasing the thermal stability required for industrial application. The immobilized α-amylase exhibited a marked increase in thermal stability as compared with its free counterpart. Immobilization of α-amylase on gold nanorods caused an increase in conformational rigidity of protein structure and limited its free the dome to undergo drastic conformational changes. This resulted in an increase in stability towards thermal denaturation [46].

Conclusion

SSF was carried out with potato peels that, served as a good substrate for the cost effective, enabling the growth of *B. lentus* and produced a considerable amount of α-amylase enzyme for both the free and immobilized enzyme. Results of this study supported the expectation that maximum enzyme stability was at pH 7.5-8, and maximum activity was observed at the temperature of 40°C. While thermo-stability of the enzyme was observed at 40–50°C. After the discussion of the results and according to reports of the previous investigators in fields related to this study, it could be claimed that *B. lentus* with the activity of ~1990 U/ gm can be considered as a good candidate for amylase production for industrial applications. In addition using of potato peels as raw material for the enzyme, synthesis could be of great commercial significance.

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