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Full Length Research Paper

Improved production, purification and some properties of α-amylase from *Streptomyces clavifer*

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Improvement of the α-amylase productivity of *Streptomyces clavifer* was achieved through studying the effect of some nutritional factors and ultra violet (UV) mutagenesis. Modification in the formula of liquid starch medium by decreasing the concentration of soluble starch to 1%, increasing the corn steep liquor to 2%, and adding 0.75% glucose and 0.01% L-valine caused significant increase in the enzyme productivity from 8640 to 23450 U/L. UV variants of high amylase productivity (54620 U/L) was isolated from the parent strain. A promising level of α-amylase production (50850 U/L) at large scale level was obtained by cultivating the tested strain in the optimum condition using laboratory fermentor of 14 L. Enzyme purification was achieved by ethanol precipitation, diethylaminoethyl cellulose (DEAE-C), and Sephadex G-100 gel filtration chromatography. The final preparation had 22.0% activity recovery and approximately 156.1 fold purification. The purified enzyme had molecular weight of approximately 50 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme exhibits maximum activity at pH 6 and 60°C and showed maximum stability at pH 6 and up to 40°C.

Key words: *Streptomyces clavifer*, α-amylase, UV mutagenesis, purification.

INTRODUCTION

Amylase enzymes play important roles in the biotechnology industries with many potential applications in food, fermentation, textile and paper (Pandey et al., 2000; Sanghvi et al., 2011). The spectrum of application of α-amylases has widened in many sectors such as clinical, medicinal and analytical chemistry (Toncova, 2006; Abou-Elela et al., 2009). Amylases can be obtained from several sources such as plants, animals and microbes. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability (Mishra and Behera, 2008; Li et al., 2011). Microorganisms have become increasingly important as producers of industrial enzymes due to their biochemical diversity and the ease of improving the enzyme productivity through environmental optimization and genetic manipulation.

There are various reports on starch degrading microorganisms from different sources and respective amylase activity (Kathiresan and Manivannan, 2006). A promising α-amylases producer *Streptomyces* strain, identified as *Streptomyces clavifer*, was isolated from Western Region of Saudi Arabia (Yassien and Asfour, 2011). In order to improve the α-amylase production by the selected isolate, knowledge regarding the nutritional factors affecting this process needs to be well identified (Boyaci, 2005; Yang et al., 2011). In addition, the α-amylases productivity of *S. clavifer* can be efficiently increased through genetic manipulation (Mishra and Behera. 2008).

The present investigation dealt with improvement of α-amylases productivity of *S. clavifer* through optimization of some physiological parameters and ultra violet (UV) mutagenesis. In addition, production of amylase enzyme by the tested strain at large scale level was carried out by using laboratory fermentor of 14 L. The effect of pH and temperature on the activity of the purified enzyme and its

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Table 1. Different culture media prepared by supplementing liquid starch media with different organic nitrogen sources to study their effects on the growth and α-amylase production by *S. clavifer*.

Nitrogen source	M1*	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Corn steep liquor	1%	2%	3%							1%	1%	1%
Peptone				1%	2%					1%		
Tryptone						1%	2%				1%	
Yeast extract								1%	2%			1%

M1* is the LS medium.

stability was also determined.

MATERIALS AND METHODS

Micro-organism

A strain of *S. clavifer*, a promising α-amylase producer micro-organism was isolated previously from soil sample in the Western Region of Saudi Arabia (Yassien and Asfour, 2011).

Quantitative determination of α-amylase production

The α-amylase activities and their specific productivity (enzyme activity/dry cell weight) were determined as described by Yassien and Asfour (2011). The inoculum of the suspension was adjusted to give a final count of 10^3 CFU/ml in a 50 ml of liquid starch medium (Linsmaier and Skoog (LS) medium; 2% soluble starch, 1% Corn steep liquor, 0.6% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 0.8% $CaCO_3$, 0.5% NaCl and pH 7) in 250 ml Erlenmeyer flask and incubated in an incubator shaker (S19R-2, Sheldon, USA) at 250 rpm and 30°C for 96 h incubation period. After incubation, a centrifugation of a known volume of culture broth at 14,000 rpm for 20 min was carried out. An aliquot of the clear supernatant was taken to evaluate the productivity of α-amylase enzyme. The residue was washed twice and the dry cell weight per liter of culture broth was then calculated.

Enzyme assay

The method was carried out as described by Swain et al. (2006). One unit of the enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue color intensity of starch iodine solution at 50°C in 1 min/ml.

Effect of different nutritional factors on the α-amylase production

The effects of different nutritional factors on the enzyme production were investigated to modify the LS medium to be suitable for maximum α-amylase productivity by the tested strain. The experiments was carried out as described earlier. Each experiment was carried out in triplicate. At the end of incubation, both cellular growth and α-amylase production was determined. Each factor that proved optimum for α-amylase production was routinely incorporated in subsequent experiments.

Effect of different organic nitrogen sources

To a group of flasks containing the LS medium, different organic nitrogen sources were added at different concentrations and the pH was adjusted to 7 (Table 1). The flasks were inoculated and incubated at 30°C for 96 h.

Effect of different carbohydrates

The effect of different carbohydrates on the production of α-amylase enzyme was studied by adding 1% of different carbon sources to the modified LS medium before adjusting the pH to 7. The flasks were inoculated and incubated at 30°C for 96 h.

Effect of addition of different amino acids

Different amino acids were incorporated into the modified LS medium at a concentration of 0.01 w/v, and the pH was adjusted to 7.0. The flasks were inoculated and incubated at 30°C for 96 h.

Improvement of α-amylase production by UV mutagenesis

Ultra violet mutagenesis was carried out to *S. clavifer* as described by Adel et al. (2006) to obtain mutants of higher α-amylase. The UV treated cells after appropriate dilution was spread onto soluble starch nitrate medium forming low count of colonies after incubation. The growth colonies were selected randomly and tested for their enzyme productivity. A control experiment was carried out in parallel in which the cells were not exposed to UV light. The exposure time and the distance between the Petri dish and UV sources that gave 99.9% kill was determined experimentally.

Large scale production of α-amylase enzyme in a laboratory fermentor

The fermentation process was carried out in a laboratory fermentor of 14 L (Bioflo 115, New Brunswick Scientific Co., Inc., NJ, USA). The working volume of the culture medium was 8 L of the optimum fermentation medium. The fermentation was conducted using batch mode. The pH of the medium was initially adjusted to 7.0 with ammonium hydroxide prior to sterilization and the fermentation was conducted without pH control. The agitation in the fermentor was carried out using two six-bladed Rushton turbine impellers rotated at 250 rpm. The incubation temperature was 30°C, and the aeration rate 5 to 10 L/min. Sterile silicone oil was added to control the foam formation. Samples were aseptically withdrawn at different time intervals during fermentation for monitoring microbial growth and α-amylase productivity.

Purification of α-amylase

The purification process was carried out as described by Ammar et al. (2002) with slight modification. All the operations of enzyme

Table 2. Effect of different organic nitrogen sources on the growth and α-amylase productivity by *S. clavifer* in LS medium at 30°C and 96 h incubation period.

Nitrogen source	Amylase productivity (U ± SD)	Dry cell weight (g)	Specific enzyme productivity (U/g)
M1*	8640 ± 145	8.9	970.8
M2	14860 ± 327	8.5	1748.2
M3	13250 ± 295	8.8	1505.7
M4	2590 ± 56	5.3	488.7
M5	3140 ± 74	5.6	560.7
M6	5820 ± 114	8.5	684.7
M7	6140 ± 122	8.7	705.7
M8	4820 ± 130	8.3	580.7
M9	3210 ± 85	6.7	479.1
M10	8730 ± 180	8.5	1027.1
M11	6580 ± 105	8.9	739.3
M12	2140 ± 87	6.7	319.4

M1* is the LS medium.

purification were performed at 4°C. Centrifugation was conducted at 7000 rpm for 20 min. In such experiment, fresh culture of the tested organism in the optimum fermentation medium was prepared. The microbial cells were removed by centrifugation, and the clear cell-free supernatant was used as starting material for α-amylase enzyme purification. The culture supernatant was first concentrated by ethanol precipitation (2 × volume), followed by dialysis against distilled water overnight and then applied on diethylaminoethyl cellulose (DEAE-C) column chromatography (3.5 × 15 cm) that was pre-equilibrated by 0.02 M sodium acetate buffer pH 6.2. The adsorbed proteins were eluted in salt gradient (with 800 ml of 0 to 1 M NaCl). The fractions that showed enzyme activity were pooled and dialyzed overnight against distilled water and then lyophilized. The concentrated enzyme solution was purified by gel filtration on Sephadex G-100 column (3.5 × 80 cm). The fractions that showed activity were pooled and again applied on DEAE-cellulose column chromatography (3.5 × 15 cm) that was pre-equilibrated by 0.02 M sodium acetate buffer pH 6.2. The elution process was performed using salt gradient (with 500 ml of 0 to 1 M NaCl). The fractions that showed enzyme activity were pooled, dialyzed and lyophilized.

Protein determination

Quantitative determination of protein was carried out as described by Bradford (1976).

Polyacrylamide gel electrophoresis

The homogeneity of the purified enzyme was demonstrated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE (Sambrook et al., 1989). The purified enzymes (final pre-paration of purification procedure) were examined by using SDS-PAGE.

Effect of pH on α-amylase activity and stability

The optimum pH of enzyme activity was determined by mixing 0.5 ml 1% starch solution in Britton-Robinson buffer (0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH adjusted to the desired pH with 0.2 M NaOH.), pH range from 3 to 9, with 0.5 ml of enzyme solution (0.27 U) and assayed for enzyme activity. The effect of pH on the enzyme stability was evaluated by preparing different samples of 0.1 ml of enzyme solution (27 U) with 0.9 ml Britton-Robinson buffer with different pH ranging from 3 to 9. The samples were stored at 4°C for 24 h and the remaining activity determined.

Effect of temperature on α-amylase activity and stability

To determine the optimum temperature of the purified enzyme, 0.5 ml of 1% soluble starch that was dissolved in Britton and Robinson's Universal buffer, pH 6.0, and 0.5 ml of the enzyme solution (0.27 U) were reacted together. The optimum temperature was measured at a range from 30 to 75°C for a 10 min reaction time. To determine themostability, 1.0 ml of the enzyme solution (0.27 U) was kept at various temperatures for 15 min and then allowed to cool down at room temperature. The remaining activity was then determined.

Statistical analysis

Statistical significance between means was tested by analysis of variance and student t-test using Instat-ANOVA software. The differences between means were considered statistically significant when the test yielded a value $P<0.05$.

RESULTS

Effect of different organic nitrogen sources

The results (Table 2) show a significant increase in the enzyme productivity by increasing the concentration of corn steep liquor in the culture medium. The highest amylase production (14860 U/L with specific enzyme production 1748.2 U/g dry cell weight) was obtained in the presence of 2% corn steep liquor. Accordingly, the formula of the LS medium was modified by increasing the

concentration of corn steep liquor to 2%.

Effect of different carbohydrates

In such experiment, different carbon additives were added to the modified LS medium (with 2% corn) in which the concentration of corn steep liquor was increased to 2%. A significant increase in the enzyme production (16440 U/L with specific enzyme productivity 1957.1 U/g dry cell weight) was obtained by adding 1% glucose (Table 3). In addition, higher enzyme production (18288 U/L with specific enzyme productivity 2078.4 U/g dry cell mass) was achieved by reducing the starch and glucose concentrations in the culture medium to 1 and 0.75%, respectively. Accordingly, the formula of the LS medium used for the next experiments was modified by increasing the concentration of corn steep liquor to 2%, decreasing the starch concentration to 1% and adding 0.75% glucose.

Effect of addition of different amino acids

According to the obtained results (Table 4), a significant increase in the α-amylase production by *S. clavifer* was only observed in the presence of L-cysteine, DL-valine, DL-isoleucine, L-arginine and L-glutathione. The highest level of α-amylase production (23450 U/L) was obtained in the presence of 0.01% DL-valine with specific amylase production of 2576.9 U/g dry cell weight. Accordingly, the highest α-amylase productivity (23450 U/L) of *S. clavifer* was achieved by using a modified LS medium (coded CGV-LS), prepared by increasing the concentration of corn steep liquor to 2%, reducing the starch concentration to 1% and adding 0.75% glucose and 0.01% valine.

Improvement of the α-amylase production by UV mutagenesis

According to the obtained results, exposure of the bacterial suspension to UV rays (256 nm) at a distance of 30 cm from the UV source for 25 min is enough to kill 99.9%. The results show that seven isolated colonies formed larger halo-forming zones than that obtained by the parent strain. For these mutants, quantitative determination of their amylase productivity was tested. The range of amylase production by selected mutants was 27500 to 54620 U/L (Figure 1). The highest amylase productivity was obtained by the mutants coded AM-7-1-4 (54,620 U/L, approximately 2.3 times more than that of the parent). Accordingly, the mutant strain coded AM-7-1-4 was selected for further studies at a large scale level using laboratory fermentor of 14 L containing 8 L of CGV-SL medium.

Large scale production of α-amylase by the selected *S. clavifer* mutant (AM-7-1-4) in a laboratory fermentor

According to the obtained results (Figure 2), α-amylase activity could be first detected after 24 h of cultivation and reaching the maximum level (50850 U/L) after about 108 h. The fermentation process was continued for 120 h where the total α-amylase productivity reached a plateau. On the other hand, maximum cell growth was reached after about 72 h of cultivation.

Purification of α-amylase enzyme

The purification of α-amylase enzyme was achieved by ethanol precipitation, DEAE-cellulose and Sephadex G-100 gel filtration and the results were summarized in Table 5. The final preparation had 22% activity recovery and approximately 156.1 fold purification. The purified α-amylase enzyme had a single protein band on SDS-PAGE with approximate relative molecular mass, 50 kDa (Figure 3).

pH and temperature characteristics of α-amylase enzyme

Regarding the pH, maximum enzymatic activity (less than 10% reduction) was obtained at pH range 5.5 to 6.5, with optimum pH at 6. In the case of enzyme stability, the preparation retained 85% or more of its enzymatic activity between pH 5.5 and 7, while the optimum pH is 6 (Figure 4). The results of the effects of temperature on the amylase activity showed that the optimum temperature for enzymatic activity is 60°C, while the thermal stability was up to 40°C and the activity was reduced by less than 10% when the temperature reached to 45°C (Figure 5).

DISCUSSION

An isolated strain identified as *S. clavifer*, a major producer of α-amylase, was isolated previously from the Western Region of Saudi Arabia. The present study focuses on improving the α-amylase productivity by *S. clavifer* through optimization of some nutritional factors and UV mutagenesis. The nutrition factors have a great influence on the production of α-amylase enzyme (Kar and Ray, 2008). The organic nitrogen sources such as beef extract, peptone and yeast extract are the common nitrogen sources for α-amylase production. Maximum α-amylase yield by *Streptomyces erumpens* was obtained when 1% of beef extract, or peptone was used as nitrogen source in the culture medium (Syed et al., 2009). Soybean meal is also a promising nitrogen source for raw starch digestive enzyme (RSDE) production due to its low

Table 3. Effect of different carbon additives on the cellular growth and α-amylase productivity by *S. clavifer* in modified LS medium (containing 2% corn steep liquor) at 30°C and 96 h incubation period.

Carbon additive	Amylase productivity (U ± SD)	Dry cell weight (g)	Specific enzyme productivity (U/g)
Control	14360 ± 285	8.2	1751.2
Glucose	16440 ± 312	8.4	1957.1
Lactose	11280 ± 182	7.8	1446.2
Dextrine	9330 ± 154	7.6	1227.6
Sucrose	11230 ± 173	8.2	1369.5
Mannitol	9810 ± 156	7.8	1257.7
Fructose	10245 ± 132	7.7	1330.5
Maltose	9735 ± 116	7.9	1232.3
1% Starch and 0.75% glucose	18288 ± 356	8.8	2078.2

Table 4. Effect of different amino acids on the cellular growth and α-amylase productivity by *S. clavifer* in modified LS medium (containing 2% corn steep liquor, 1% starch, and 0.75% glucose) at 30°C and 96 h incubation period.

Amino acid	Amylase productivity (U ± SD)	Dry cell weight (g)	Specific enzyme productivity (U/g)
Control	18350 ± 671	8.6	2133.7
hydroxyproline	19110 ± 713	8.3	2302.4
L-cysteine	21230 ± 825	9.2	2307.6
Glycine	17830 ± 585	9.4	1896.8
DL-valine	23450 ± 882	9.1	2576.9
L-alanine	18210 ± 725	8.8	2069.3
L-proline	18250 ± 613	8.6	2122.0
L-aspartic acid	18310 ± 547	8.9	2057.3
DL-ornithine	18180 ± 564	8.8	2065.9
L-glutamic acid	14590 ± 392	9.1	1603.2
L-B-phenylalanine	17890 ± 615	8.1	2208.6
DL-isoleucine	20870 ± 734	8.5	2455.3
L-arginine	21320 ± 713	8.9	2395.5
DL-serine	19340 ± 637	8.4	2302.4
L-leucine	18650 ± 574	8.7	2143.7
Glutathione	21270 ± 693	9.1	2337.4
L-methionine	18370 ± 536	8.5	2161.2
L-Glutamine	18750 ± 492	8.3	2259.0
L-cystine	13580 ± 216	8.6	1579.1
DL-tyrosine	16230 ± 356	8.2	1979.3

cost and availability. High α-amylase production by *Streptomyces aureofaciens* 77 was obtained by using 2% of corn steep liquor as a sole nitrogen source (Shatta et al., 1990). The same results were obtained in the present study, since the highest amylase productivity was obtained when 2% corn steep liquor was added to the LS medium.

In respect of the carbon sources, various raw starches and soluble starch are the preferred choices of carbon source for most micro-organisms because of their better induction on amylase production and low cost (Li et al., 2010). Raw starch such as wheat bran enhances the amylase production by *S. erumpens* (Kar et al., 2008). Also the α-amylase production by *S. aureofaciens* 77 was improved by increasing the concentration of starch as carbon source from 1 to 3% (Shatta et al., 1990). In the present study, the most suitable main carbon source was soluble starch, but the amylase productivity was increased by adding 0.75% glucose and reducing the starch concentration to 1%. However, Okolo et al. (1996)

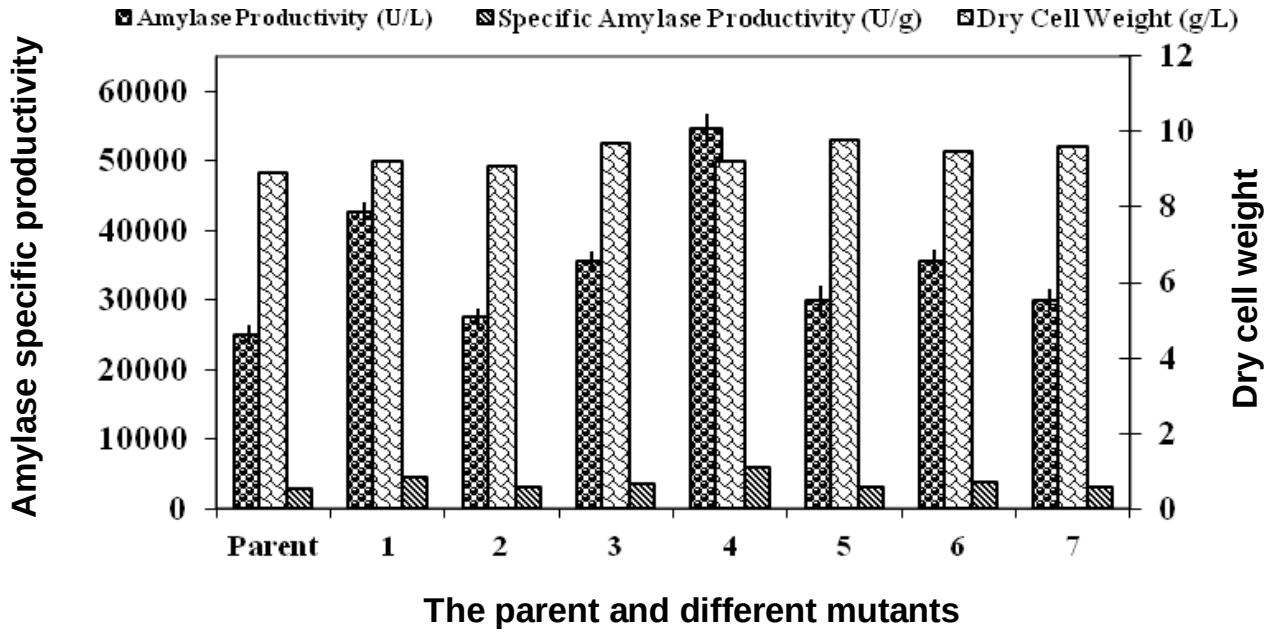

Figure 1. The cellular growth and α-amylase productivity by the selected *S. clavifer* mutants (AM-7-1-1 to AM-7-1-7) as compared to that of the parent strain.

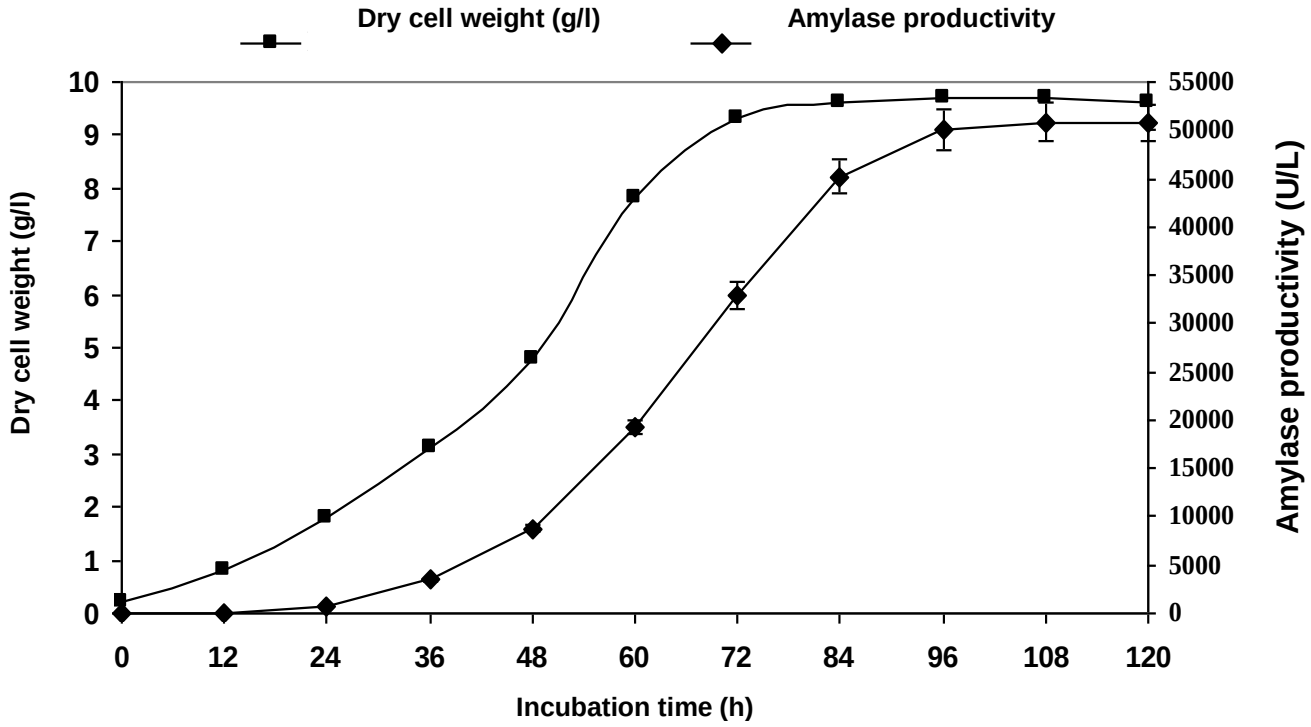

Figure 2. Kinetics of cellular growth and α-amylase productivity by the selected *S. clavifer* mutant (AM-7-1-4) along 120 h at 30°C in CGV-LS medium using a laboratory fermentor of 14 L.

reported that amylase production may be subjected to catabolite repression by glucose and other readily metabolizable substrates.

Proteinaceous nitrogen compounds, such as corn steep liquor and yeast extract, usually serve as sources of amino acids. However, the addition of pure amino

Table 5. Purification steps of α-amylase enzyme from *S. clavifer* culture supernatant.

Purification step	Total protein (mg)	Total activity (U)	Specific acivity (U/mg)	Yield (%)	Purification fold
Crude extract	26880	24220	0.9	100	1.00
Ethanol precipitation	960	23960	17.6	98.9	19.6
DEAE-cellulose	580	14520	25.0	60.0	27.8
Sephadex G-100	210	6460	30.7	26.7	34.1
DEAE-cellulose	38	5340	140.5	22.0	156.1

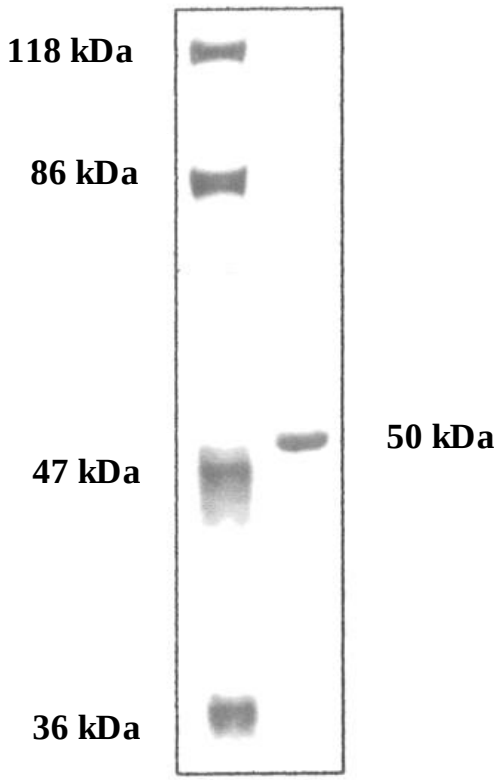

Figure 3. SDS-PAGE of purified α-amylase enzyme (20 µg) produced by *S. clavifer*. Lane 1: Molecular mass standards (β-galactosidase, 118 kDa; Bovine serum albumin, 86 kDa; ovalbumine, 47 kDa; carbonic anhydrase, 36 kDa), Lane 2: purified α-amylase enzyme produced by *S. clavifer*.

acids to the fermentation medium is occasionally required to improve the yield of some biotechnological products (Stanbury et al., 2000). In the present study, a significant increase in the α-amylase production by *S. clavifer* was only observed in the presence of DL-valine, L-arginine, DL-isoleucine, and L-glutathione. The highest level of amylase production (23450 U/L) was obtained in the presence of 0.01% DL-valine. Other types of amino acids such as L-histidine and L-hydroxyproline improve the α-amylase productivity by *Streptomyces aureofasciculus* and *Streptomyces galilaeous*, respectively (Poornima et al., 2008).

According to the obtained results, a new formula of fermentation medium coded CGV-LS medium (LS medium containing 1% soluble starch, 2% corn steep liquor, 0.75% glucose, and 0.01% valine). The level of amylase production in the new formulated culture medium CGV-LS medium is approximately 2.6 times more than that produced in the LS medium.

Improvement of enzyme production can also be done by genetic manipulation (Abo Shanab et al., 2003). Traditionally, most industrial strain improvement programs have been based on empirical random mutation and selection. However, rational selection procedures are more efficient than random screening for the selection of improved protease producing strains. Accordingly, in the present study, an attempt was made to improve the amylase production by selecting UV variants of high enzyme productivity. After exposure of the microbial suspension to UV for 25 min, different UV variants with enzyme productivity higher than that obtained by the parent strain was observed. The highest amylase productivity was obtained by the mutant coded AM-7-1-4 with enzyme productivity of 54,600 U/L which is approximately 2.3 times more than that of the parent.

The obtained results of a fermentation process in a shaking flask (25 ml) could be helpful as a guide for studying large scale enzyme production by the mutant coded AM-7-1-4 using a laboratory fermentor of 14 L. The process of large scale fermentation was carried out using the CGV-LS medium (8 L working volume). Fortunately, the optimum fermentation condition used in small scale was suitable for use in large scale fermentor with maximum enzyme production 50,850 U/L. These results could be helpful as a preliminary data for further studies on the selected strain at industrial level.

The α-amylase activity was affected by different environmental factors, such as pH and temperature. On studying the effect on pH and temperature on the enzyme activity, it was observed that the optimum condition was pH 6 and 60°C. Sufficient activity (≥ 86%) was also observed at pH range 5.5 to 6.5 and reaction temperature ranged from 50 to 65°C, respectively. These results are in agreement with previous studies of Ammar et al. (2002) and Kaneko et al. (2005). On the other hand, Yang and Wang (1999) reported that the optimum temperature of α-amylase enzyme activity from *Streptomyces rimosus* ranges between 35 and 45°C.

Regarding enzyme stability, the optimum condition of

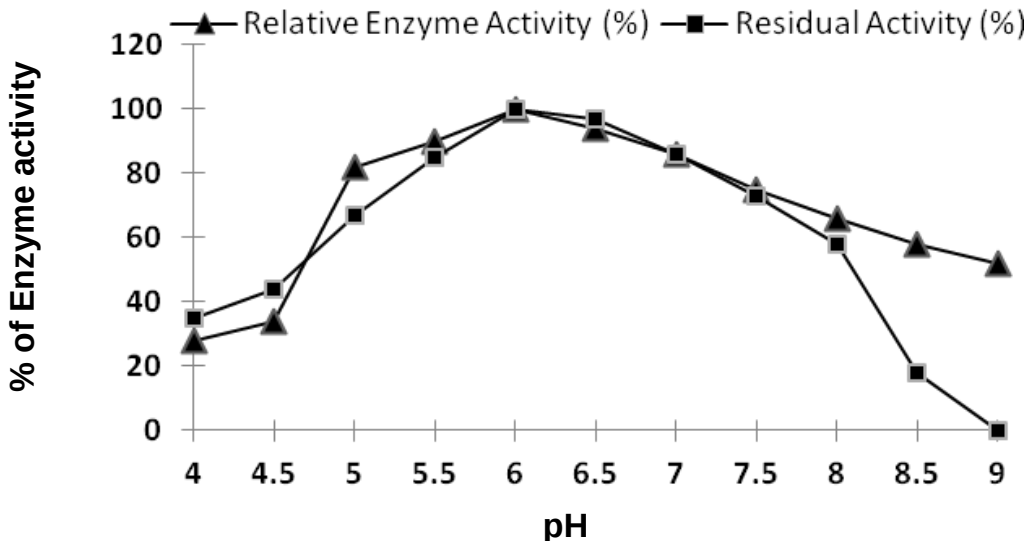

Figure 4. Effects of temperature on the α-amylase activity and stability. The remaining activity after treatment of the enzyme at different temperature was measured by incubating at 50°C and pH 6 for 10 min.

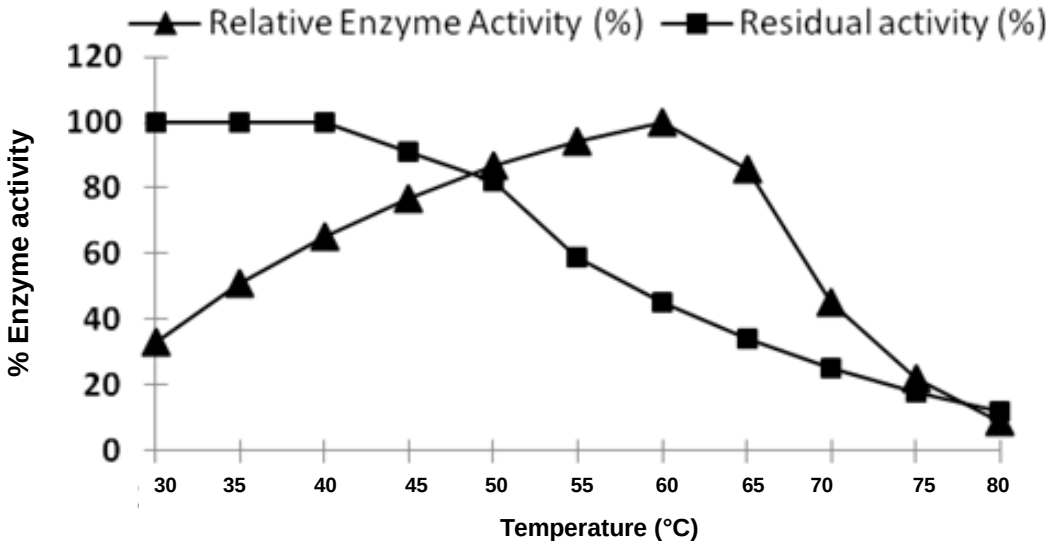

Figure 5. Effects of pH on the α-amylase activity and stability. The remaining activity after treatment of the enzyme at different pH was measured by incubating at 50°C and pH 6 for 10 min.

enzyme stability were pH 6 and up to 40°C. The same thermal stability was reported by Ammar et al. (2002). Higher thermostable α-amylase enzyme (up to 50°C) was reported by Kaneko et al. (2005) with the same optimum pH. On the other hand, wider pH range (5 to 7) for α-amylase stability was investigated by Ammar et al. (2002). According to the obtained results, a high level of α-amylase production by *S. clavifer* can be obtained at large scale level. Accordingly, it can be used as initial step for application at industrial level. In addition, optimum condition (pH 6 and 60°C) of the enzymatic reaction is helpful in achieving the highest level of enzyme activity.

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