Production and Evaluation of Alpha-Amylase Produced From *Bacillus Amyloliquefaciens*

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Abstract

This research was carried out to determine the optimal environmental and nutritional factors for amylase production and its activity by bacterial strains *Bacillus amyloliquefaciens* as well as to evaluate the kinetic characterization of the produced α-amylase. From the obtained results showed that the optimum pH, inoculum size, fermentation period, incubation temperature, carbon and nitrogen sources for produced glucose and amylase activity were 7.0, 1500 µl /ml, 72 h, 37ºC, starch and tryptone, respectively when the basal broth medium was used as a fermentation medium rather than the using of starch broth medium for the production of α-amylase. In addition, data showed that the shaking method was better than the static one for amylase production. Moreover, the optimal conditions for fermentation process gave a higher records of produced glucose and amylase activity rather than each factor individually. The values of amylase activity, protein content and specific amylase activity were 697.60 U/ml, 57.14 mg/ ml and 12.21U /mg protein, respectively.

Concerning the factors affecting on produced α- amylase activity were evaluated. From the obtained results , the optimum temperature and pH values of the produced α-amylase from *Bacillus amyloliquefaciens* were found to be 65 ºC and 6.0, respectively and the reaction activity were reached their maximum values were 15.46 U/ml /min for temperature and 18.8 U/ml/min for pH. The V_{max} and K_m values of the production enzyme under investigation were determined by incubation fixed amount of enzyme with varied concentrations of soluble starch at 65 ºC , pH 6.0 and 15 min were 32.3 U/ml/min and 1.596 ml/100 ml, respectively . On the other hand, the obtained resulted indicated that the metal ion concentration of 1 mM had a greater effect on enzyme activities than 5 mM. The relative activities of the produced α-amylase were activated by Fe^{3+}, Cu^{2+} and Ca^{2+} at 1 mM, but strongly inhibited by Mn^{2+} and Ni^{2+} at both 1 mM and 5 mM concentrations.

Key words: α-amylase, *Bacillus amyloliquefaciens*, production, fermentation, optimization, kinetic parameters.

Introduction

Enzymes are an important class of proteins produced by living cells of microorganisms, plants and animals to catalyze specific biochemical reactions of the metabolic pathways of the cells. Among the produced enzymes, amylases are the most important group for biotechnology and account approximately 65% of enzyme market in the world **(Balkan and Figen, 2007 and Abd-Elhalem *et al.*, 2015).**

Among them, α-amylase (E.C.3.2.1.1) is a calcium metalloenzymatic that hydrolysis the internal α-1, 4-glycosidic linkages in starch and leads to the fermentation of low molecules weight oligosaccharides as glucose, maltose and maltotriose units **(Aiyer, 2005).**

Microbial enzymes such as amylases are widely used in industrial processes due to their low cost, large productivity, chemical stability, environmental protection, plasticity and vast availability (**Mishra and Behera, 2008).**

Today, amylases find potential widespread application in different industrial processes especially in food industry for liquefaction and saccharification of starch into fructose and glucose syrups **(Khusro *et al.*, 2017)**.

Konsoula and Liakopoulou-Kyriakides (2007) found that bacterial species *B. cereus* and *B. subtilis* have been explored for production of amylases enzyme. Also, *B. licheniformis*; *B. stearothermophilus* and *B. amyloliquefaciens* were considered as good producers for the thermostable α-amylase**.**

Ashwini *et al.*, (2011) investigated the optimization, production and partial purification of extracellular α-amylase from *Bacillus* sp. *Marini*. They found that the enzyme production was find maximum in presence of starch as carbon source, yeast extract as nitrogen source, 6.5 % NaCl concentration, temperature 40°C and pH 7.0. At the optimum conditions *B.* sp. *Marini* produced 8000 U of amylase.

Deb *et al.*, (2013) studied the production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. They found that maximum enzyme production was obtained after 48 h of incubation in a fermentation medium with initial pH 9.0 at 42°C under continuous agitation at 15 rpm. The size of inoculum was also optimized which was found to be 1% (v/v). Enzyme production was 2.43 times higher after optimizing the production conditions as compared to the basal media. Studies on crude amylase revealed that optimum pH, temperature and reaction time of enzyme activity were 6.5, 60°C and 40 min, respectively.

Karnwal and Nigam (2013) indicated that the heat stable α-amylase produced from *B.*

amyloliquefaciens was the first liquefying enzyme used on a large commercial scale.

Saha *et al.*, (2014) found that the specific activity of amylase produced by *B. amyloliquefaciens* was 13.5 U/mg.

Onofre *et al.*, (2016) evaluated of α-amylase produced by the endophytic strain of *Penicillium* digitatum in solid state fermentation (SSF) and submerged fermentation (SMF). They found that the maximum yield of the enzyme was observed with SSF, using rice bran as substrate after 72 h of fermentation, with 1,625 U/mL. The α-amylase had an optimal pH at 6.5 and optimal temperature at 37°C. All the ions resulted in a decrease in the activity of α-amylase in the concentration of 5 mM.

Rasmy (2018) studied the kinetic properties of α-amylase produced by *Bacillus megaterium* RAS103 under optimum conditions in submerged fermentation. He found that amylase activity was maximized to 106.39±2.36U/ml under the optimized culture conditions of a fermentation medium of 2% starch supplemented by 3g/L yeast extract, adjusted at pH 8.0, inoculated with 1% bacterial inoculum and incubated at 35°C for 24h. The V_{max} and K_m values of the produced amylase were 81.30U/ml and 0.878mg/ml, respectively for hydrolysis of starch in a reaction mixture of pH 6.0 at 45°C for 20min.

The purpose of this work was to study the optimization of cultural conditions such as carbon sources, pH, temperature, nitrogen sources for α-amylase production by *B. amyloliquefaciens.* In addition to kinetic properties for the produced α-amylase were evaluated.

Materials and Methods

B.amyloliquefaciens was obtained from Agric. Microbiology Department, Faculty of Agric., Ain Shams Univ., Egypt. *B.amyloliquefaciens* strain was sub-cultured on nutrient agar medium pH 7.0 **(Taha, 1964)** for purification, then maintained as a stock culture at 4-5°C in refrigerator for subsequent studies.

Initial experimental for detection of α-amylase

B. amyloliquefaciens was tested for amylase production using plate assay method by employing clear zone technique **(Atlas *et al.*, 1995)** on starch agar medium **(Harrigan and McCance, 1976)**.

Assay of α-amylase activity

Amylase activity was assayed by the method described by **Miller (1959)**.

Inoculum preparation

Spores of *B. amyloliquefaciens* were transferred to a 100 ml conical flask containing 25 ml nutrient broth medium **(Taha, 1964)**. The flask was kept on shaker (150 rpm) at 37ºC for 24 h. One ml from homogenous bacterial suspension contains about 80 $\times 10^5$ colony forming unit / ml was used as a standard inoculum.

Production medium of α-amylase

Production of α-amylase by *Bacillus amyloliquefaciens* was carried out using basal medium according to **Bose and Das (1996).**

Optimization of pH, inoculum size, fermentation period, temperature, carbon and nitrogen sources on α-amylase enzyme productivity and enzyme activity

Effect of pH on enzyme production and enzyme activity was studied by adjusted at different pH values (6.0, 6.5, 7.0, 7.5 and 8.0), production medium was inoculated by *B. amyloliquefaciens* with different inoculum sizes namely 100, 500, 1000, 1500 and 2000 µl/ml. Similarly, the effect of fermentation period was studied by adjusted different incubation periods (24, 48, 72, 96 and 120 h.). The inoculated medium was incubated at different incubation temperatures (33, 35, 37 and 39°C). Also, the effect of carbon, nitrogen sources was studied by adjusting different carbon sources (glucose, lactose, sucrose, dextrin and starch), nitrogen sources (beef extract, yeast extract, urea, tryptone, potassium nitrate and sodium nitrate) in the production medium.

Effect of fermentation medium on amylase production by *B. amyloliquefaciens*

Either basal broth medium pH 7.0 **(Bose and Das, 1996**) or starch broth medium pH 7.0 **(Harrigan and McCance, 197**6) were incubated at 30°C for 72 h using static incubator.

Effect of static and shaking methods on amylase production

Basal broth medium (pH 7.0) was inoculated by *B. amyloliquefaciens* (1500 µl / ml) 24 h. The inoculated medium was divided into two groups, the first was incubated using shaking incubator (150 rpm) while, the other was incubated using static incubator at 30°C for 72 h **(Sundarram and Murthy, 2014).**

Effect of optimal conditions on amylase production

Basal broth medium (pH 7.0) amended with the best carbon and nitrogen sources and inoculated by *B.amyloliquefaciens* (1500 µl / ml) 24 inoculated medium was incubated at 37 ºC using shaking incubator (150 rpm) for 72 h.

At the end of incubation period in all previous experiments, the cultures were centrifuged at 5000 rpm for 15 min at 4°C to remove the bacterial cells. Then, the supernatant was collected and α-amylase enzyme was assayed using the method described by **Miller (1959)** as abovementioned.

Determination of protein content and specific activity of the produced α- amylase

Protein content and specific activity of the produced α-amylase were determined in extracted crude enzyme according to the method described by **Bradford (1976)** using bovine serum albumin (BSA) as a standard.

Characterization of the produced α-amylase

Produced amylase from the experiment of optimal conditions was used to study the factors affecting on the enzyme activity such as temperature, pH, substrate concentration, sodium chloride and metal ions were evaluated. Optimum temperature for enzyme activity was determined by incubating crude enzyme substrate reaction mixture at different temperatures i.e: 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C for 15 min using shaking incubator and assaying the enzyme activity as mentioned before **(Miller, 1959).**

The effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.1 M (pH 4.0-5.5, acetate buffer), (pH 6.0-7.5, phosphate buffer) and (pH 8.0- 9.0, Tris-Hcl buffer). The relative activities of the produced α-amylase were estimated.

Effect of substrate concentrations on the produced α- amylase activity different concentrations from soluble starch (0.4 ,0.8, 1.2 ,1.6 ,2.0 ,2.4 ,2.8 ,3.2 ,3.6 ,4.0% w/v) were investigated for detection of amylase kinetics properties. The reaction mixture contains extracted amylase was incubated at optimum temperature 65ºC, pH 6.0 for 15 min using shaking incubator. The reaction velocity and activity of the produced α-amylase were estimated by Michael's-Menten equation following by using Lineweaver-Burk plots for calculate K_m and V_{max}.

Effect of sodium chloride concentrations on the produced α- amylase activity was investigated. The extracted enzyme was mixed with different concentrations of sodium chloride (0. 5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6 % w/v), pH (6.0), soluble starch at a concentration of 2.8% was added as a substrate. Then, the reaction mixture was incubated at optimum temperature 65 ºC for 15 min using shaking incubator.

Effect of metal ions concentration on amylase activity was investigated the reaction mixture contains the extracted enzyme was mixed with different metal ions i.e: Fe^{++}, Cu^{++}, Ca^{++}, Mn^{++} and Ni^{++} at two concentrations (1 and 5 mM from each ion) , pH (6.0) and soluble starch (2.8%) a substrate . Then, the reaction mixture was incubated at optimal conditions as mentioned above. At the end of incubation period in all above experiments for evaluation of enzyme activity, the produced α- amylase was assayed using the method described by **Miller (1959)** as abovementioned.

Results and Discussion

Initial experimental for detection of α-amylase production

Fig. (1) show that the bacterial strain *Bacillus amyloliquefaciens* was able to hydrolyze starch as a result of its ability for amylase production showing zone of hydrolysis around the colonies on starch agar medium supplemented with soluble starch. In this concern, some species of genus *Bacillus* produces a large variety of extracellular enzymes such as amylases, which are considerable for industrial importance **(Swain *et al.*, 2006, Deb *et al.*, 2013).**

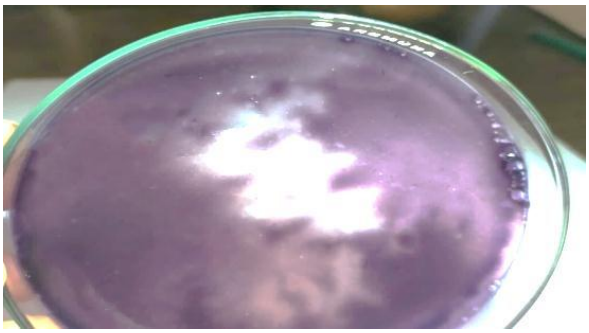

Fig. (1): Zone of clearance due to the hydrolysis of starch by *B.amyloliquefaciens.*

Effect of initial pH on α-amylase production by *B.amyloliquefaciens*

Data in Table (1) indicate that Obtained glucose and amylase activity were higher when the pH of fermentation medium ranged from 6.5-7.0 compared to other investigated pH values. The highest values of produced glucose and amylase activity were found to be 55.81 mg/ml and 31.01 IU/ml/min, respectively when the initial pH of fermentation medium was 7.0. While, the lowest records of abovementioned parameters were observed when the initial pH of fermentation medium was 6.0. α-amylase production by bacteria significantly dependens on the medium pH because it effects on the growth and many metabolic reactions as well as the movement of molecules across cell membrane. The obtained results are in accordance with **Sundarram and Murthy (2014).** Many studies revealed an optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and amylases production because bacteria required neutral pH for optimum growth **(Gangadharan *et al.*, 2006 and Gupta *et al.*, 2008)**.

Table1. α-amylase production by *B. amyloliquefaciens* at different pH values.

pH values	Obtained glucose	Amylase activity
6.0	34.55	19.19
6.5	53.55	29.75
7.0	55.81	31.01
7.5	48.10	26.72
8.0	47.4	26.35

Effect of inoculum size on α-amylase production by *B.amyloliquefaciens*

Table (2) show that the effect of inoculum size. From the obtained results the produced glucose and amylase activity were increased with the increasing of inoculum size of *B.amyloliquefaciens* to reach their maximum values 57.16 mg/ml and 31.75 IU/ml/min respectively when the fermentation medium was inoculated with 1500 µl /ml. Similar results were observed by **Deb *et al.*, (2013)**.

Table 2. α-amylase production by *B. amyloliquefaciens* using different inoculum sizes.

Inoculum size (µl/ml)	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
100	34.81	19.34
500	36.04	20.02
1000	39.56	21.98
1500	57.16	31.75
2000	54.70	30.39

Effect of fermentation period on amylase production by *B.amyloliquefaciens*

Results in Table (3) show that the records of produced glucose and amylase activity were increased with the increasing of incubation period to reach their maximum values (98.51 mg/ml and 54.73 IU/ml/min, respectively) at 72 h incubation period. Further increase of incubation time significantly decrease the enzyme production. This is may be due to nutrienteed efficiency of the culture medium and accumulation of toxic metabolites.

These results are in accordance with those observed by **Asgher *et al.*, (2007)**. **and Haq *et al.*, (2010).** They found that the highest activity of α-amylase production by *Bacillus subtilis and B. amyloliquefaciens* was recorded at 37°C after 48 h incubation period.

Table 3. α-amylases production by *B. amyloliquefaciens* at different fermentation periods.

Fermentation periods (h)	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
24	42.76	23.76
48	45.25	25.14
72	98.51	54.73
96	72.06	40.03
120	69.56	38.65

Effect of nutritional medium on α-amylase production by *B. amyloliquefaciens*

Obtained results in Table (4) clearly indicate that, produced glucose and amylase activity were higher (92.50 µg/ml and 51.38 IU/ml/min) when the basal broth medium (77.80 µg/ml and 43.20 IU/ml/min) was used as a fermentation medium rather than the using of starch broth medium. The decrease in amylase biosynthesis when used starch for fermentation medium might be due to inaccessibility of dissolved oxygen to the bacterial cells because of the high viscosity of carbon source in the fermentation medium.

Table 4. α-amylase production by *B. amyloliquefaciens* at different nutritional media.

Fermentation medium	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
Starch broth medium	77.80	43.20
Basal broth medium	92.50	51.38

Effect of static and shaking methods on α- amylase production

Data in Table (5) clearly indicate that the shaking method gave a higher values (116.95 µg/ml and 64.97 IU/ml/min, respectively) for produced glucose and amylase activity by bacterial strain *B.amyloliquefaciens* as compared to static method. Therefore, the shaking method was used in all subsequent experiments that Performed in the current study. Similar results was observed by **Sundarram and Murthy (2014).**

Table 5. α-amylase production by *B. amyloliquefaciens* using shaking and static methods.

Fermentation methods	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
Shaking	116.95	64.97
Static	75.96	42.20

Effect of incubation temperature on amylase production by *B.amyloliquefaciens*

Data in Table (6) show that produced glucose and amylase activity were increased with the increasing of incubation temperature and reached their maximum records at 37ºC. The highest values of produced glucose and amylase activity were 119.59 µg/ml and 66.44 IU/ml/min, respectively. These results are in agreement with reported by **Nusrat and Rahman (2007) and Haq *et al.*, (2010).** The decrease in amylase activity was obtained at higher temperature (39 ºC) might be due to inhibition of cell division and growth as well as protein denaturation of bacterial cells **(Oyeleke *et al.*, 2011).**

Table 6. α-amylase production by *B. amyloliquefaciens* at different incubation temperatures.

Incubation temperature (^{O}C)	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
33	84.52	46.96
35	102.47	56.93
37	119.59	66.44
39	53.38	29.65

Effect of carbon sources on α-amylase production by *B.amyloliquefaciens*

Data in Table (7) show that the highest records of produced glucose and amylase activity (122.94 µg/ml and 68.30 IU/ml/min, respectively) were observed when starch was used as a carbon source. Whereas, the lowest values of abovementioned parameters were observed when lactose was used as a carbon source in fermentation medium. In general, obtained data in Table (7) clearly indicated that the oligosaccharides such as dextrin and polysaccharides such as starch gave a higher records of abovementioned criteria compared with either monosaccharides or disaccharides (glucose, lactose and sucrose). In this concern, starch was known to increase amylases production by *B. subtilis* **(Sodhi *et al.*, 2005 and Gupta *et al.*, (2008).**

Table 7. α-amylase production by *B. amyloliquefaciens* using different carbon sources.

Carbon sources	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
Glucose	109.74	60.97
Lactose	115.90	64.39
Sucrose	77.77	43.21
Dextrin	115.90	64.39
Starch	122.94	68.30

Effect of nitrogen sources on amylase production by *B.amyloliquefaciens*

Generally, the obtained data in Table (8) clearly indicate that the organic nitrogen sources (beef extract, urea, yeast extract and tryptone) gave a higher records produced glucose and amylase activity compared with the inorganic nitrogen sources (potassium nitrate and sodium nitrate). The highest values of produced glucose and amylase activity were 125.57 µg/ml and 69.76 IU/ml/min, respectively when tryptone was used as a nitrogen source in fermentation medium. Whereas, the lowest values of abovementioned parameters were observed with sodium nitrate. Different inorganic and organic nitrogen sources were observed by **Haq *et al.*, (2010), Demirkan, (2011)**, **Sundar *et al.*, (2012), Simair *et al.*, (2017) and Abdullah *et al.*, (2018)**

Table 8. α-amylase production by *B. amyloliquefaciens* using different nitrogen sources.

Nitrogen sources	Obtained glucose (µg/ml)	Amylase activity (IU/ml/min.)
Beef extract	104.75	58.20
Urea	102.41	56.89
Yeast extract	111.79	62.11
KNO_3	96.25	53.47
$NaNO_3$	91.85	51.03
Tryptone	125.57	69.76

Effect of optimal conditions on amylase production by *B.amyloliquefaciens*

The comparison between amylase production by *B.amyloliquefaciens* using basal broth fermentation medium and the same fermentation medium with the optimal environmental and nutritional conditions that obtained in the abovementioned experiments in the current study and the obtained results are tabulated in Table (9).

From the obtained data showed that produced glucose and amylase activity were highly increased and the values were (125.57 µg/ml and 69.76 IU/ml/min, respectively) at optimal conditions versus basal medium broth, the values were 53.55 µg/ml and 29.75 IU/ml/min, respectively.

From the abovementioned results, it could be concluded that the enzyme production was 2.34 times higher after optimization the production conditions compared to the using of each factor individually in basal medium. These results are in good agreement with those reported by **Deb *et al.*, (2013).**

Table 9. α-amylase production by *B. amyloliquefaciens* using optimal conditions.

Basal medium broth (g/l)		**Optimal conditions (g/l)**	
Starch, soluble	10.0	Starch, soluble	10.0
Tryptone	2.0	Tryptone	2.3
KH_2PO_4	1.0	KH_2PO_4	1.0
Na_2HPO4	2.5	Na_2HPO4	2.5
NaCl	1.0	NaCl	1.0
$(NH_4)_2SO_4$	2.0	$(NH_4)_2SO_4$	2.0
$MgSO_4.7H_2O$	0.05	$MgSO_4.7H_2O$	0.05
$CaCl_2$	0.05	$CaCl_2$	0.05
pH	6.5	pH	7.0
Incubation	30ºC	Incubation	37 ºC
Fermentation period	48 h.	Fermentation period	72h.
Inoculum size	1000µl	Inoculum size	1500 µl
Estimated parameters			
Parameters	Value	Parameters	Value
Obtained glucose	53.55	Obtained glucose	125.57
Amylase activity	29.75	Amylase activity	69.76

Determination of protein content and specific activity for amylase

Protein content of the produced α-amylase was determined and the obtained results were illustrated in Table (10). From these data, it can be clearly indicate that the values of amylase activity, protein content and specific amylase activity were found to be 697.60 U/ml, 57.14 mg/ml and 12.20 U/mg protein, respectively. The obtained results are similar with those reported by **Mahdavi *et al.*, (2010)** who found that the protein content of amylase was 50 (mg/ml) .While **Saha *et al.*, (2014)** found specific activity of amylase was 13.50 U/mg.

Table 10. Protein content and specific activity of α-amylase.

Enzyme activity (U/ml)	**Protein content (mg/ml)**	**Specific activity (U/mg**
697. 60	57.14	12.21

Evaluation and characterization of the produced α-amylase enzyme

The major goal of this trial is to study the influence of various parameters such as temperature, pH, substrate concentration, sodium chloride concentration and metal ions concentration on the reaction or relative activity of the produced α-amylase enzyme.

Effect of temperature

Fig. (2) clearly indicate that the relative amylase activity was gradually increased with the increasing of incubation temperature . The value of amylase activity was reached its maximum (100 %) at 65ºC. The lowest record of amylase activity was observed at 30ºC of incubation period. The decrease of enzyme activity at low temperatures is due to the decrease in atomic motion which decreases the activation energy of the reaction between the substrate and enzyme molecules. Also, the decrease of enzyme activity at high temperatures might be due to thermal denaturation of the enzyme **(Krishma and Radhathirumalaiarasu, 2017).** Similar results were observed b**y Kumar *et al.*, (2011)** who found that the 70 ºC was the optimal temperature for maximum amylase activity. Also, **Deb *et al.*, (2013)** found that the optimal temperature for amylase activity was observed at 60ºC. Moreover, **Simair *et al.*, (2017**) found that the maximum amylase activity was at 75 ºC. In addition**, Abdulaal (2018)** reported that the maximum activity of α-amylase was at 50 º C**.**

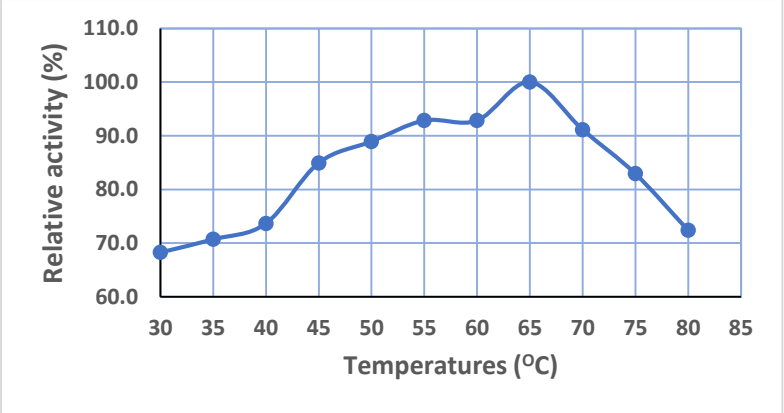

Fig.(2): Relative activity of the produced α-amylase at different incubation temperatures.

Effect of pH

Data in Fig. (3) show that the relative α-amylase activity was gradually increased with the increasing of pH to reach its maximum record 100% at pH 6.0 While, obtained results clearly indicate that the records of relative amylase activity was gradually decreased after pH 6.0 to reach their minimum record 56% at pH 8.0 .These results are in accordance with the findings of **Coronado *et al.*, (2000)**, **Alva *et al.*, (2007)**, **Asad *et al.*, (2011)** and **Bole *et al.*, (2013).** The amylase activity is obviously affected by the pH of the reaction medium, this is because the binding of substrate and enzyme is frequently dependent on charges distribution on broth of them **(salman *et al.*, 2016)**

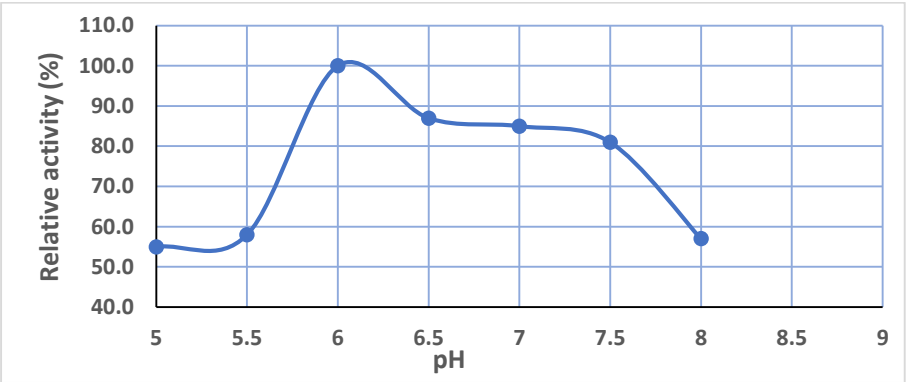

Fig. (3): Relative activity of the produced α-amylase at different pH values.

Effect of substrate concentration

Substrate concentration is one of the most important factors which affect the reaction activity and reaction velocity of enzyme reaction. It is clear that from results in Fig. (4), the reaction activity of the produced α-amylase was increased with the increasing of starch concentrations and reached its maximum value at 2.8 % then gradually decreased. The increase in substrate concentration at a constant enzyme concentration lead to an increase in amylase activity until reaching a saturation point, this is might be due to the saturation of active sites of enzyme molecules with substrate molecules, as well as the period of the time necessary for a new substrate molecule to hit a free bond decrease constantly until the enzyme is surrounded by many substrate molecules to fill a bond immediately after having released the product.

Regarding to the kinetic parameters of α-amylase produced by *Bacillus amyloliquefaciens* .The maximum reaction velocity (V_{max}) for the produced α-amylase with varied concentrations of soluble starch as a standard substrate (0.4 to 4.0%) was determined and equaled to 32.3 U/ml/min. While, the Michael's - Menten constant (K_m) was found to be 1.596 ml/100ml which can be obtained by the half point of V_{max} at saturation curve (Fig.4.)

Also, V_{max} and k_m were determined by **Lineweaver and Burk, (1954)** technique. When plotting $1/V_o$ against 1/(S), a straight was obtained (Fig.5).

The slope of this line represents $K_m/V_{max,}$ which obtained as 0.0494 and the intercept on the Y-axis corresponds to $1/V_{max}$ was found to be 0.031, so the intercept on X-axis to $1/K_m$ was estimated to 0.627. From the obtained data for V_{max} and K_m values using Lineweaver -Burk plots equation were almost equall to the results obtained in the saturation curve as shown in Fig (4). There results are in agreement with those reported by **Haq *et al.*, (2010), Demirkan (2011) and Rasmey (2018).**

Table 11. Effect of substrate concentration (%) on the reaction activity and velocity of the produced α-amylase.

Substrate concentration (%)	[1/s]	Reaction activity (units/ml/min)	Reaction Velocity (v)	[1/v] x10^{2-}
0.4	2.50	5.6	6.5	15.4
0.8	1.25	8.0	10.8	9.3
1.2	0.83	11.7	13.9	7.2
1.6	0.63	17.0	16.2	6.2
2.0	0.50	19.0	18.0	5.6
2.4	0.42	21.4	19.4	5.2
2.8	0.36	32.3	20.6	4.9
3.2	0.31	30.2	21.6	4.6
3.6	0.28	26.9	22.4	4.5
4.0	0.25	20.6	23.1	4.3

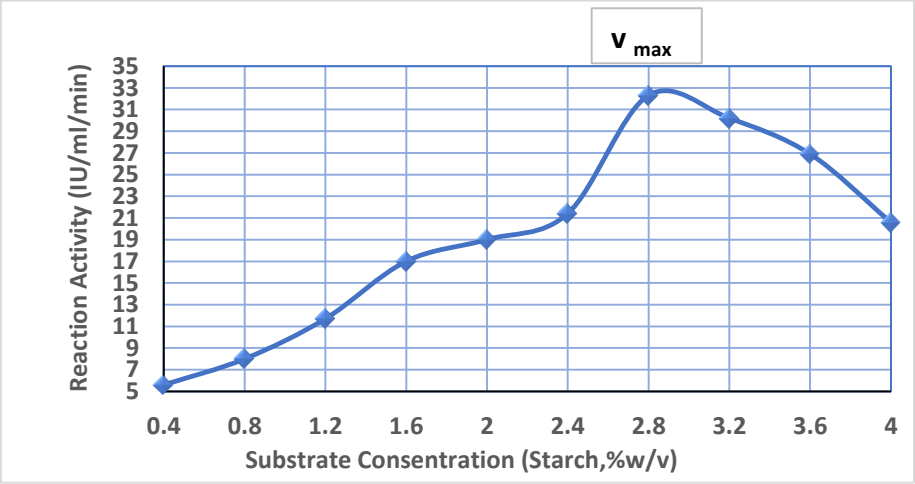

Fig .(4) : Effect of substrate concentrations on reaction activity of α-amylase.
V_{max} = 32.3Units/ml/min K_m = 1.596ml/100ml

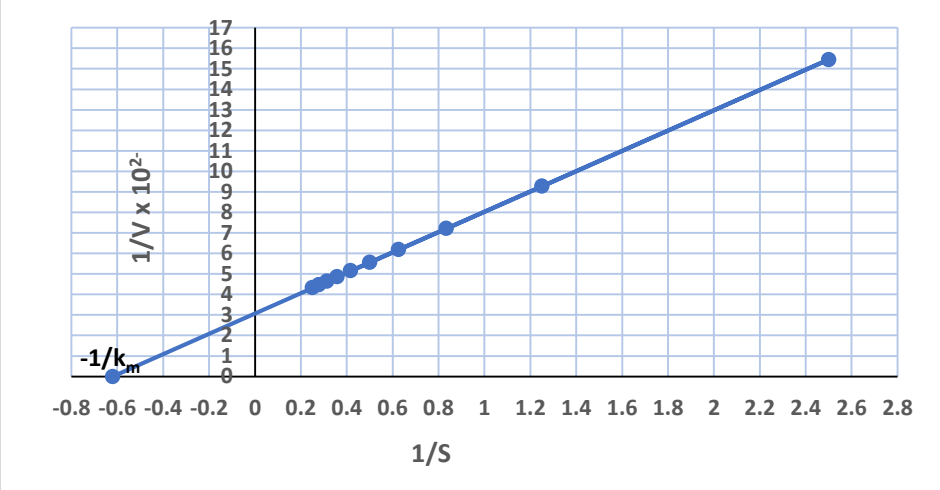

Fig. (5): Lineweaver -Burk plots for calculating V_{max} and K_m of the produced α- amylase by *Bacillus amyloliquefaciens*.

Effect of sodium chloride concentration

Concerning the effect of sodium chloride concentrations on amylase produced by *Bacillus amyloliquefaciens* was illustrated in Fig (6). From the obtained results showed that the relative amylase activity was gradually increased with the increasing of sodium chloride concentration to reach their maximum records at 5 %. In view of the obtained results, it could be mention that 78.6 and 93.1% of amylase activity were retained in mixture reaction at 4.0 and 4.5 % NaCl concentrations respectively. These results are in accordance with **Khire and Pant**

(1992), Amoozegar *et al.*, (2003), Ashwini *et al.*, (2011) and **Bajpai *et al.*, (2015).** While, **Arabaci and Arikan (2018)** found that the maximum α-amylase activity produced by *Bacillus subtilis* N8 was found to be 3% NaCl concentration.

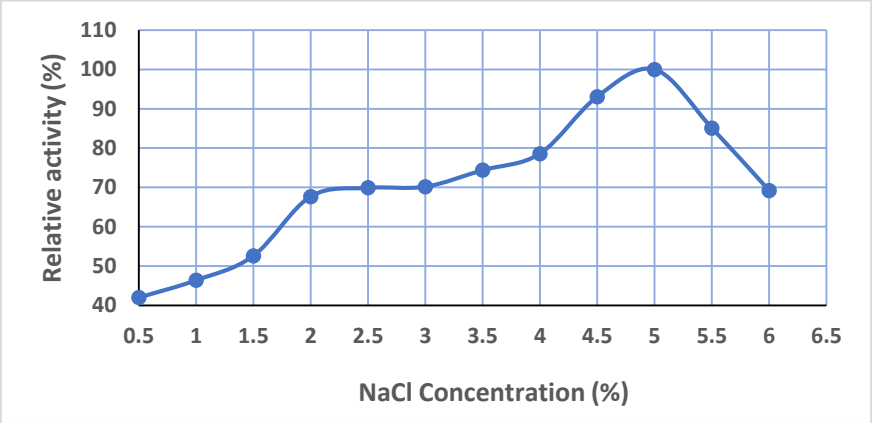

Fig. (6): Relative activity of the produced α-amylase under different concentrations of NaCl .

Effect of metal ions concentration

Table (11) and Fig. (7) illustrated the effect of metal ions on relative activity of the produced α-amylase . Generally , obtained results clearly indicate that amylase relative activity was higher at a concentration 1 mM for all tested metal ions (Fe^{+3}, Cu^{+2} , Ca^{+2} , Ni^{+2} and Mn^{+2}) compared to the concentration of 5 mM from abovementioned metal ions. In addition, data in Fig. (7) show that metal ions Fe^{+3} , Cu^{+2} and Ca^{+2} at a concentration of 1 mM increased the relative activities of amylase by 13.02, 42.34 and 40.09 % , respectively compared to the control . While, metal ions Ni^{+2} and Mn^{+2} at the same concentration decreased the relative activities of amylase by 13.72 and 37.53 %, respectively compared to the control. Regarding the retained relative amylase activity under different tested metal ions at a concentration 5mM, data in Fig. (7) clearly observed that 92.24, 82.24 and 40.11 % from relative amylase activity were retained for Cu^{2+} , Ni^{2+} and Mn^{2+} , respectively compared to the control .

On the other hand, it is importance to mention that the Ca^{+2} ion at a concentration 5mM increased the relative activity of amylase by 32.45% as compared to the control. The increase of relative activity of amylase in presence of high concentration of Ca^{+2} ion could be attributed to the Ca^{+2} ion is considered metal activator for amylase also, amylase is a calcium metalloenzymatic. **(Aiyer, 2005 and Behal *et al.*, 2006).** These observation of effect metal ions concentrations were reported by several researches**, Gangadharan *et al.*, (2009), Annamalai *et al.*, (2011), Demirkan (2011),**

Onofre *et al.*, (2016) and **Abdulaal (2018)** they found that metal ions at low concentrations increased the activity, but at high concentrations the activity was decreased.

Table 12. Relative activity of α-amylase under different metal ion concentrations.

Metal ions	Concentrations (mM)	Relative activity (%)
Control	None	100.00
Fe^{+3}	1	113.02
Cu^{+2}	1	142.34
Ca^{+2}	1	140.09
Ni^{+2}	1	86.280
Mn^{+2}	1	62.470
Fe^{+3}	5	82.580
Cu^{+2}	5	92.240
Ca^{+2}	5	132.45
Ni^{+2}	5	82.240
Mn^{+2}	5	40.110

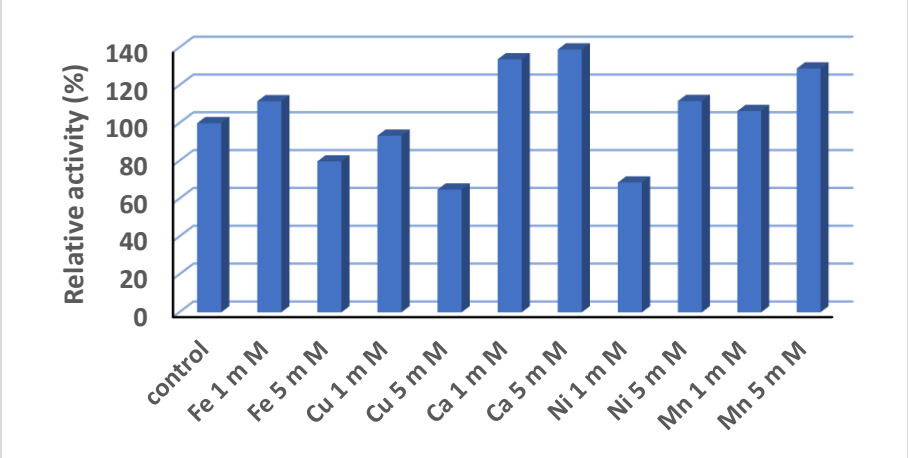

Fig. 6. Relative activity of amylase under different metal ion concentrations.

Conclusion

In view of the obtained results, *Bacillus amyloliquefaciens* able to produces α-amylase. It is important to mention that, the environmental and nutritional conditions for α-amylase production by *Bacillus amyloliquefaciens* were detected. In addition, about 72.90 % of relative activity for the produced α-amylase was retained after incubation the crude enzyme mixture at 80°C for 15 min. Also, it was found that the produced enzyme tolerate a high temperature which can be used in an industrial processes such as bakery, beer and sugar industries and the production of glucose and fructose syrups.

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إنتاج وتقيم إنزيم الألفا أميليز المنتج من بكتريا الباسيليس أميلوليكوفشينس

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يهدف هذا البحث لتحديد الظروف البيئية والظروف الغذائية المثلي لإنتاج إنزيم الفا–أميليز بواسطة بكتريا الباسيليس أميلوليكوفشينس، كذلك تم دراسة الظروف التي تؤثر علي درجة النشاط الانزيمي المنتج والتي تشمل تأثيرات كل من درجة الحرارة و درجة الحموضة وتركيز مادة التفاعل (النشا الذائب) وتركيز كلوريد الصوديوم وكذلك تركيز بعض الأيونات المعدنية.

ولقد أظهرت النتائج المتحصل عليها أن أنسب الظروف البيئية من درجة حموضة ، فترة تحضين ، درجة حرارة ، لإنتاج إنزيم الأميليز كانت هي علي التوالي 7، 72 ساعة ، 37 درجة مئوية ، بينما أوضحت النتائج أن كل من النشا والتربتون هما أفضل مصادر للكربون والنيتروجين لإنتاج إنزيم الأميليز بواسطة بكتريا الباسيليس أميلوليكوفيشينس. كما أوضحت النتائج أن استخدام طريقة الرج في عملية التخمير لإنتاج الإنزيم قد أعطت إنتاجاً أعلي مقارنة باستخدام طريقة التخمير الثابت.

بجانب ذلك ، فإنه عند إنتاج إنزيم الأميليز باستخدام الظروف المثلي التي تم التحصل عليها من الدراسة ، فقد أظهرت النتائج أن معدل الانتاجية يزيد بمقدار 2.34 مرة تحت الظروف المثلي لنشاط الإنزيم المنتج وقد تم تقدير النشاط للإنزيم الخام المنتج ومحتواه من البروتين الإنزيمي ودرجة النشاط النوعي والقيم المتحصل عليها هي 697.6وحدة / ملليتر ، 57.14 مليجرام / ملليتر و 12.20 وحدة دولية /مليجرام بروتين علي التوالي.

بينما عند دراسة العوامل التي تؤثر علي نشاط الإنزيم الخام المنتج ، أظهرت النتائج أن أعلي معدل للنشاط النسبي لإنزيم الأميليز لوحظ عند درجة حرارة 65 درجة مئوية ، درجة حموضة 6 ، كما وجد أن السرعة القصوي لأقصي معدل نشاط إنزيمي هي 32.3 وحدة /مللي/دقيقة بينما قيمة ثابت ميكاليس منتن هي 1.596 مللي/100مللي . وكذلك فإن نشاط الإنزيم قد زاد تدريجياً بزيادة تركيز كلوريد الصوديوم في مخلوط التفاعل حيث وصل إلي أقصاه عند تركيز 5% . بينما تأثير الأيونات المعدنية (الحديديك ، النحاسوز ، الكالسيوم ، النيكل والمنجنيز) علي نشاط الإنزيم المنتج فقد أوضحت النتائج أن نشاط الإنزيم كان أعلي معدل لها في وجود التركيز المنخفض من هذه الأيونات 1 ملليمول بالمقارنة بتركيز 5 ملليمول .

الخلاصة :

من خلال هذه الدراسة والنتائج المتحصل عليها والتي أدي الي إمكانية تحديد الظروف البيئية والتغذوية لإنتاج إنزيم الألفا أميليز من بكتريا الباسيلبيس أميلوليكوفيشينس وجد أن الإنزيم المنتج يتحمل درجة حرارة عالية ، حيث يمكن إستخدامه في المجالات الصناعية ، بجانب ذلك تم تقيم الظروف المثلي لنشاط الإنزيم المنتج لإستخدامه في عمليات التسكر للنشا لإنتاج شراب الجلوكوزوالفركتوز .