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Production, Purification and Characterization of Alfa-amylase produced by Bacteria from Pharaonic Lake

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Abstract:

In the present study thirty bacteria isolates collected from Pharaonic lake, Menoufiya Governorate, Egypt. Out of which 12 isolates that were α-amylase producer. Isolate **No**. 1 was selected as the best enzyme producer, identified as ***Alloiococcus otitis*** and was used for further studies. Attempts were made to optimize the cultural condition for getting high yields of enzyme. The optimum level of pH for enzyme production was 8.0 on Bennett medium after 8 hours. Among carbon sources, maltose supported maximum production of α-amylase followed by lactose, sucrose, starch and manitol. Urea was the best nitrogen source for enzyme production, followed by L-asparagine. The purified fraction detected by the appearance of a single band corresponding to 38 k Da in SDS-PAGE, and the enzyme was purified 67.0-fold with amylase units 225 ml and specific activity of 234.4 U/mg. The pure enzyme was stable at all degrees of temperatures (20–70ºC). The maximum activity was at pH of 9.0. Starch was the best substrate. CaCl2, MgCl2 and KCl were the best activators. There is a continuous decreasing in the activity due to the increase of SDS and H_2O_2 concentrations. Enzyme was stable in presence of butanol, n-hexane and toluene.

KEY WORDS: α-amylase, Bacteria, Purification.
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1. Introduction:

Amylases are a class of enzymes that are capable of digesting these glycosidic linkages found in starches. Amylases can be derived from a variety of sources. They are present in all living organisms, but the enzymes vary in activity, specificity and requirements from species to species and even from tissue to tissue in the same organism. Raw-starch digesting amylases are produced by a variety of living organisms, ranging from microorganisms including fungi, yeast, and bacteria to plants and humans .

The α-amylase enzyme produced by many microorganisms like *B.amyloliquefaciens* [1]. *Bacillus licheniformis* [2] *Chromohalobacter sp TVSP-101* [3]; *Bacillus polymyxa, B.mesentericus,B.vulgarus, and B.megaterium* [4]. *Bacillus licheniformis GCB-U8* [5]; *Bacillus sp.PS-7* [6], *Bacillus licheniformis M27* [7]. *Halobacillus sp. MA-2* [8]; *Halomonas meridiana* [9], *Bacillus cereus MTCC1305* [10], and *Rhodothermus marinus* [11]. *Monascus sanguineus* also has not been explored for its efficiency to produce amylase enzymes under solid state fermentation, various substrates were screened and among them beetroot as a solid substrate has given maximum yield (0.029 U/mL). Enzyme activity was further optimized by response surface methodology (RSM) and maximum experimental yield of 0.014 U/mL was obtained at optimized conditions of pH 5, incubation temperature of 50° C and 10 min incubation time. AMATLAB software package was used for the graphical and regression analysis of the experimented data. Enzyme kinetics was calculated with different concentrations of starch and observed Km value was 0.055 mM from linear regression analysis. The enzyme was moderately inhibited (44.7%) by NaCl and KCl (0.105 U/mL) with minimum inhibition (14.8%) observed with SDS. Molecular weight calculation and amylase confirmation in protein sample was done by SDS–PAGE and Zymography. Calculated molecular weight was 56 k Da. Alkaline amylase produced

by *M. sanguineus* has exhibited high efficiency towards removal of stains on cloths in combination with commercial detergent (Surf excel) at 20°C. [12].

From mutant micelial strain *Aspergillus niger* 33-19 CNMN FD 02A, through alcohol ethylic precipitation of cultural liquid, amylolytic preparation Amilonigrin AS was isolated with 10x degree of purity and a specific activity of 138.3U/mg proteins. α-Amylase from 20mM Tris-HCl extract of Amilonigrin AS was purified to homogeneity by PD-10 column gel filtration and HiTrap TMQ column ion exchange chromatography. A trial for the purification of α-amylase resulted in an enzyme specific activity of 199.68U/mg protein with purification fold 8.9. The analyses of purified α-amylase for molecular weight was carried out by SDSPAGE electrophoresis, with revealed two polypeptide bands estimated to be 66 and 40.5k Da, probably being two α-amylase isoforms. [13].

Arthrobacter psychrolactophilus ATCC 700733 grew with a doubling time of 1.5–2.3 h (22°C) and produced up to 0.2 units/mL (soluble starch assay) of extracellular amylase in tryptic soy broth without dextrose (TSBWD) containing 0.5% or 1.0% (w/v) soluble starch or maltose as the fermentable substrate. Time course experiments in media containing soluble starch as substrate showed that amyl lytic activity appeared in cultures at 24 h (after exponential growth had ceased) reached peak levels in 72–96 h, and declined rapidly after reaching peak levels. Peak levels were highest in TSBWD containing 1.0% soluble starch. Photolytic activity appeared at about the same time as amyl lytic activity and increased during the period of amylase production. Significant amylase production was not observed in cultures in TSBWD with 0.5% glucose or in cultures grown at 28°C, but low levels of amylase were observed in TSBWD cultures grown at 19–23° C which contained no added carbohydrate. A single band of activity was observed after electrophoresis of supernatant fractions in non-denaturing gels, followed by in situ staining for amyl lytic activity. The amylase possessed a raw starch-binding domain and bound to uncooked corn, wheat or potato starch granules. It was active in the Phadebas assay for a-amylase. Activity was maximum on soluble starch at a temperature between 40°C and 50°C. The amylase after purification by affinity chromatography on raw starch granules exhibited two starch-binding protein bands on SDS gels of 105 k Da and 26 k Da [14].

The production, purification, and characterization of alfa-amylase enzymes isolated from a new bacterial isolate isolated from water was the aim of this work.

2. Material and methods:

Isolation of Bacteria:

Water samples from Pharaonic Lake, Minoufiya Governorate, Egypt, were collected according to standard methods for examination of water and wastewater [15]. One ml of each sample was spread onto the surface of the nutrient agar medium [16], and the plates were incubated for 24 hours at 30ºC. Colonies were checked for purity by repeated sub-culturing and the pure colonies selected were maintained on slants of the same medium and stored at 5ºC.

Preparation of inoculum:

A single colony of the experimental organism was grown on nutrient agar plates

The pH of the medium was adjusted to 7.0. Then from these plates, 250 ml Erlenmeyer flasks containing 50 m l starch nitrate liquid medium were inoculated and incubated for 24 h. on a rotary shaker at 250 rpm and 30ºC. Two ml ($1x10^8$spore/ml) of this inoculum was used to inoculate 250 m l Erlenmeyer flasks containing 50 ml of production medium.

Protein determination:

Protein of all enzymatic preparations was determined by the Lowery method [17].

Screening for alfa-amylase production:

All thirty tested isolates were screened for their ability of α-amylase production**,** the organisms were inoculated on starch nitrate culture medium plates. contained [18], 324 (gl): soluble starch, 20.0 ; KNO ,1.0; K HPO ,0.5; -1 MgSO4 2 4 2 .7H O, 0.5 ; FeSO4 .7H O, 0.05; and agar, 20 ; sea water or lake water 1L. Incubation at 30°C was carried out for 24 hour after which the plates were 2 flooded with Gram's iodine solution (0.1% I and 1% KI) and the colonies with the largest halo-forming zone were chosen for further investigations . [19].

Alfa amylase activity:

The α-amylase activity was determined according SKB modified method [20, 21]. The reaction mixture (15) contained 10ml of buffered (0.2M acetate buffer, pH 4.7) 1% soluble starch (Sigma) solution as the substrate and 5ml adequately diluted enzyme sample. Incubation was performed at 30°C for 10 minutes. The reaction was terminated and developed with iodine solution in ratio 0.5ml reaction mixture/50ml iodine solution (5mg iodine and 50mg KI in 100ml 0.1N HCl) for 5min. One unit of activity was defined as the amount of enzyme, which catalyzes hydrolysis of one-gram soluble starch up to dextrin with different molecular weight; hydrolysis range 30% under the assay conditions. The absorbance of reaction mixture will be determined at 546 nm on spectrophotometer. The absorbance will be then converted to mg of maltose from the standard curve established with maltose.

Optimization of culture conditions for Alfa-amylase production:

To find out the best α-amylase by the isolate No. 1, we study the effect of different media, incubation period, pH, temperature, carbon, and nitrogen sources.

Purification of the enzyme:

In order to purify α-amylase enzyme, to be used further for the kinetics, study the culture broth was first centrifuged at 10,000 rpm for 30 min. The enzyme activity in the supernatant was measured. The supernatants were precipitated by ammonium sulfate (60 %) and activity was measured again. Total protein concentration in the enzyme solution was determined by Lowery, s method [17]. After ammonium sulfate precipitation, the enzyme sample was

dissolved in Tris-HCl buffer pH 8.0 and further purified by carboxy-methyl (CM) Sephadex ion exchange chromatography. The molecular weight of the purified fraction of the enzyme was then determined by Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method [22].

Effect of different concentrations of substrate on alfa- amylase activity:

Reaction mixture were designed varying (from 1 % to 3.5 %) from starch-iodine stock solution to which 125-µl enzyme solution for the isolate No.1 was added then, the enzyme activity was measured as mentioned above.

Effect of enzyme concentrations on alfa- amylase activity:

For the isolate **No.1**, substrate concentration of 3.5 % starch-iodine solution (125 µl) was added to the enzyme concentrations (250, 500, 750, 1000, 1250, 1500, 1750 and 2000 µl**)**, incubation was performed at 37°C for 10 min. After incubation the enzyme activity was measured as mentioned above.

Effect of temperature on Alfa- amylase activity:

The influence of temperature on the catalytic activity of the α-amylase was determined by incubating sample (125 µl) of the pure enzyme of isolate **No. 1** in Eppendorf tubes at 20, 30, 40, 50 and 60ºC for 1 hour. The tubes were quickly cooled in ice and enzyme activity was measured.

Effects of some metal ions on Alfa- amylase activity:

Activity and stability studies were performed after the addition of each metal ion solutions sample of the purified enzyme of isolate **No. 1** were incubated for 60 minutes with 5 mM of Fe Cl_2, K Cl, Ca Cl_2, Cu SO_4, Mn Cl_2, Zn SO_4 and Mg Cl_2. Afterwards, the enzyme activity was measured.

Organic solvent-stability of pure enzyme:

Three milliliters of crude α-amylase (4.5 U/ml) of isolate **No. 1** was incubated with 1.0 ml of organic solvent at 40ºC with a constant shaking at 150 rpm for 30 min. For control, the solvent was replaced by distilled water. The relative activity which remained after 30 min. of incubation in 25% (v/v) of organic solvent was measured. The organic solvents chosen in this study were toluene, acetone, butane and n-hexane. Thereafter α-amylase activity was measured.

Effect of surfactants and oxidants on Alfa-amylase activity:

The compatibility of α-amylase with surfactants and oxidants was studied in the presence of 0.5, 1.0 and 1.5% SDS (Sodium dodecyl sulfate) as surfactants and 0.5, 1.0 and 1.5 % of H_2O_2 as oxidants. The enzyme of isolate No. 1 (4.5 U/ml) was incubated at 40ºC with surfactants and oxidants for 30 min. and thereafter the activity was measured as standard assay condition. The enzyme activity of a control sample (without surfactants and oxidants) was considered as 100%.

Characterization and Identification of the selected bacteria:

For complete identification of the most α-amylase producer isolate, several morphological, physiological and biochemical properties were examined. The identification of the best organisms for producing α-amylase enzyme were done at Micro Analytical Center, Faculty of Science, Cairo MIRCEN, Faculty of Agriculture, ASU BiologDG (2013). Biolog Gp Data Base. Relase 15G Hayward, CA: Biolog, according to Bergey´s manual of systematic bacteriology [23].

Table (1): α-amylase production by Some of the thirty bacteria isolates:

No. of isolates	Zone of Hydrolysis (mm)	No. of isolates	Zone of Hydrolysis (mm)
1	22	7	8.5
2	18	8	10
3	21	9	3
4	10	10	1.5
5	8	11	7
6	5	12	6

3. Results:

Thirty bacteria isolates were isolated. The isolates were purified by continuous sub-culturing on nutrient agar media. The pure isolates were stored in 20% glycerol at 20°C. These isolates were evaluated for their ability to utilize starch. Isolates no.1, 2 and 3 were found to produce larger clear zones (22, 18 & 21 mm) than the other isolates as shown in table 1. The isolate No. 1 was chosen as the experimental organism and was characterized in detail.

1.Effect of different media on Alfa-amylase production:

Results represented in figure (1) indicated that Bennett medium was considered to be the best medium for the α-amylase production.

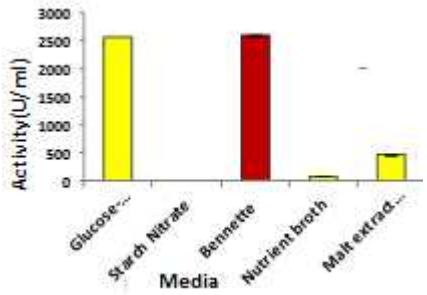

Fig (1): Effect of different media on α-amylase production

2.Effect of different incubation period on α-amylase production:

Data illustrated in figure 2 indicated that the isolate was able to produce the highest yield of α-

amylase after 8 hours incubation.

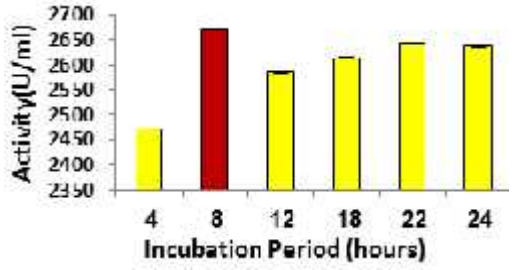

Fig (2): Effect of incubation period on the production of α-amylase

3.Effect of different carbon sources on α-amylase production:

The data obtained in figure 3 showed that maltose was the best carbon source for α-amylase production by bacteria isolate No. 1.

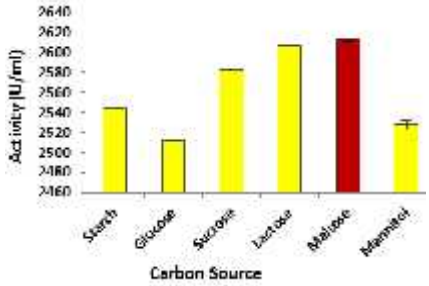

Fig (3): Effect of different carbon sources on α-amylase production by bacteria isolate No. 1.

4.Effect of different nitrogen sources on α-amylase production:

The data obtained in figure 4 showed that Urea was considered to be the best nitrogen source for the highest α-amylase productivity for bacterial strain isolate (No. 1) followed by ammonium sulfate and L-asparagine for α-amylase production by bacteria isolate **No. 1**.

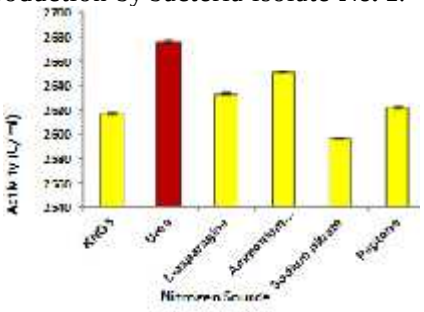

Fig (4): Effect of different nitrogen sources on α-amylase production by bacteria isolate No. 1

5.Effect of different pH values on α-amylase production:

The effect of different pH values on α-amylase production by isolate (No. 1) was represented in figure 5. Activity was found to be a maximum at pH 8.0. Below or above this optimal pH; the activity gradually decreased as compared to that at the optimal pH value recorded for the enzyme production blue single band appeared corresponding to 38 k Da as shown in figure7.

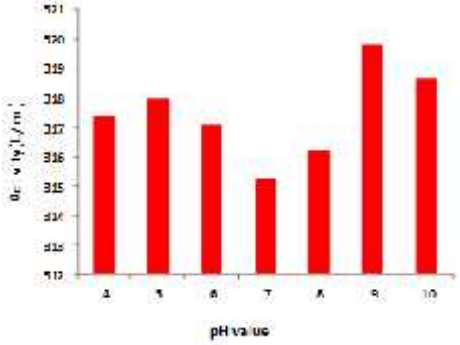

Fig (5): Effect of different pH values on α-amylase production by isolate No. 1

Purification Methods:

The purified fraction from the strain was proved by the appearance of a single band corresponding to 38 k Da in SDS-PAGE as shown in Fig. (7)

α-amylase activity was eluted in fractions (3-7) with maximal value in fraction No. 5 and 6 as shown in figure 6.

The results obtained in table 2 showed that the different steps of purification for alfa-amylase with % recovery and fold purification. The enzyme was purified 67-fold with amylase units 225 ml and specific activity of 234.4 U/mg.

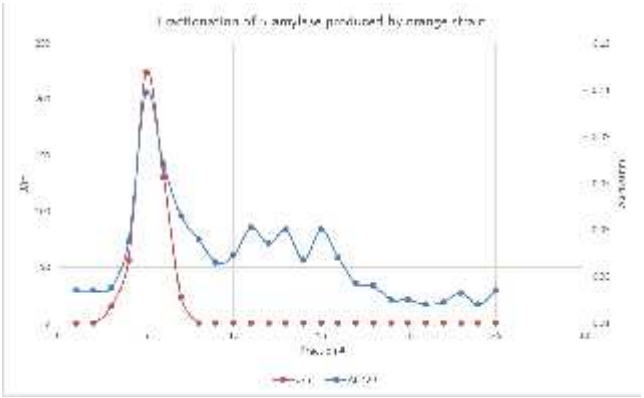

Fig (6): Fractionation of α-amylase produced by isolate No.1 with (CM) sephadex ion-exchange

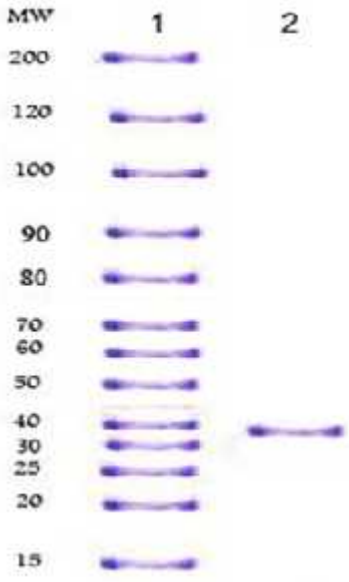

Fig (7): SDS-polyacrylamide gel stained with commissar brilliant blue, in lane (1), the molecular weight marker, in lane (2) the purified α-amylase produced by isolate No. 1 on Carboxy-methyl (CM) Sephadex ion-exchange chromatography.

Table (2): Purification of α-amylase produced by bacteria isolate No. 1

Purification step	Mg protein/ml	Amylase Units/ml	Total volume (ml)	Total units	Specific activity	Fold purification
Cell supernatant	3.55	12.5	90	1125	3.5	1.0
Pellets after $(NH_4)_2SO_4$ ppt.	4.51	276	2	552	61.2	17.5
Pellets after dialysis	2.24	247	2	494	110.3	31.5
Gel filtration (Sephadex G75)	0.96	225	2	450	234.4	67.0

1-Effect of temperature on α-amylase activity:

Results represented in figure 8 show that, the enzyme was stable at all degrees of temperature of the purified enzyme of isolate No. 1.

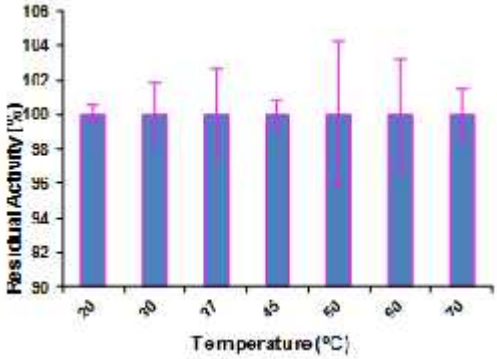

Fig (8): Effect of temperature on the activity of the purified enzyme

2-Effect of different substrate concentrations on α-amylase activity:

The results in figure 9 show that there was continues increasing g of enzyme activity due to the increase of substrate concentrations (Starch at the concentration 3.5 %), where the activity reached to 809.66U/m l. above this concentration the α-amylase activity decreased.

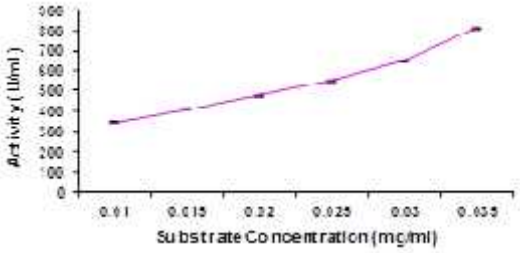

Fig (9): Effect of substrate concentrations (Starch) on the hydrolytic action of the purified enzyme

3- Effect of different enzyme concentration on α-amylase activity:

There was a continues increasing of enzyme activity due to the increase of enzyme concentrations until at concentration 2000 µl, where it reached up to (724.30) U/ml as shown in figure 10.

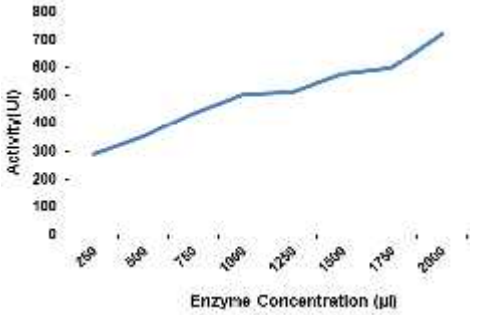

Fig (10): Effect of enzyme concentrations on the activity of the purified α-amylase enzyme

4-Effect of different metallic ions on α-amylase activity:

Results represented in figure 11 show that, CaCl2, MgCl2 and KCl were the best activator for the purified α-amylase produced by isolated No. 1. On the other hand, the other tested metallic ions gave a variable inhibition for α-amylase activity.

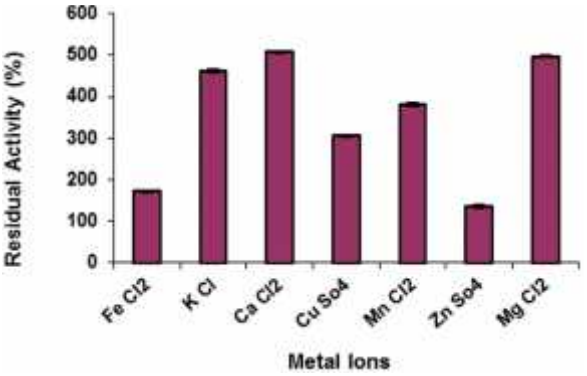

Fig (11): Effect of metal ions on the activity of the purified α-amylase enzyme

5- Effect of surfactants and oxidants on α-amylase activity:

Results represented in figure 12 show that there is a continue decreasing residual activity due to the increase of SDS and H_2O_2 concentration for enzyme produced by isolate No. 1, where residual activity reached to 83% at concentration (0.5%). Also in regard to effect of H_2O_2 concentration, there was continues decreasing of residual activity due to the increase of H_2O_2 concentration, where residual activity reached to 93% at concentration of 0.5%.

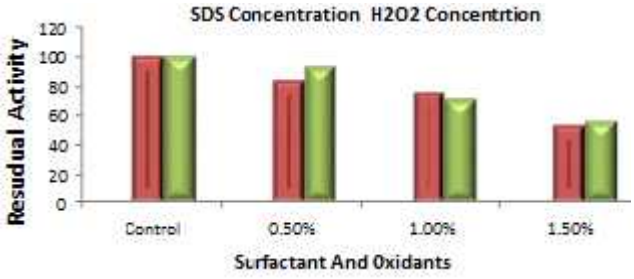

Fig (12): Effect of SDS and H_2O_2 concentrations on the activity of the purified α-amylase enzyme

6- Effect of different solvents on α-amylase activity:

Results represented in figure 13 show that the enzyme produced by isolate No. 1 was stable in presence of butanol, n-hexane and toluene.

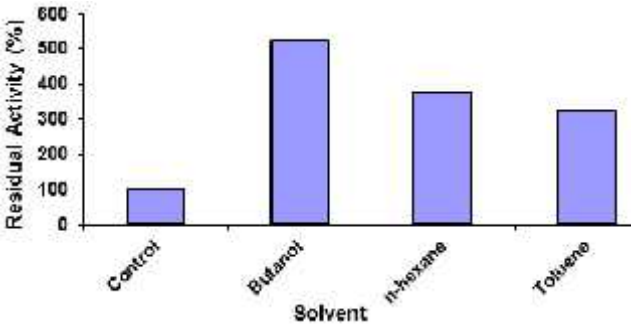

Fig (13): Effect of different solvents on the activity of the purified α-amylase enzyme

Characterization and identification of the selected Bacteria:

Out of thirty isolates of bacteria, organism No. 1 was the most active for α-amylase production. It was subjected to further studies in order to be characterized and identified.

The results obtained in Table (3,4 and 5) showed that isolate No.1, utilized various carbon sources for growth as α –D-Glucose, D-Maltose, D-Trehalose, D-Cellobiose, Sucrose, L-Rhamnose, Rhafinnose , D-Fructose, D-Turanose, Gentiobiose, Stachyose, α -D-Lactose , D-Melibiose, β-Methyl-D-Glucoside, N-Acetyl-D-Glucosamine, N-Acetyl-β-D-Mannosamine, N-Acetyl-D-Galactosamine, D-Mannose, D-Galactose, 3-Methyl Glucose, D-Fucose, L-Fucose, D-Sorbitol, D-Arabitol, Glycerol, Glycyl-L-Proline,D-Glucose-6-PO4, but D-Mannitol and Myo-Inositol were not utilized. Also, culture utilized varies nitrogen sources for growth as L-Arginine and L-Alalnine but Potassium Tellurite was not utilized and Gelatin, Dextrin, D-Salicin, Inosine, L- Serine and Pectin were observed, Degradation tolerate up to (1 % - 8%) NaCl. The spore chains were in coccus form, gram positive stain, culture is not susceptible to Rifamycine SV, Troleandomycin and L-Histidine. Growth is best on Bennett at 30 °C. The fatty acid compositions of the isolate examined is shown in Table (3, 4 and 5). The fatty acid profiles revealed that Nalidixic Acid (603), Citric Acid (459) were the predominant fatty acids.

Table (3): Physiological characteristics of isolate No.1

Test	Reaction of selected stain
Hydrolysis of:	
Gelatin	- ve/+ ve (21)
Dextrin	- ve/+ ve (0)
D-Salicin	+ ve (227)
Inosine	+ ve (188)
D-Serine	- ve (146)

L- Serine	+ ve (144)
Pectin	- ve/+ ve (12)
Production of:	
Tetrazolium Violet	- ve Sensitive (236)
Tetrazolium Blue	- ve Sensitive (197)
Growth in the presence of (% w⁄v):	
Sodium Butyrate	+ ve (726)
Lithium chloride	+ ve (450)
Sodium Bromate	-ve (256)
Sodium chloride (1%)	+ ve (736)
Sodium chloride (4%)	+ ve (599)
Sodium chloride (8%)	+ ve (525)
Sodium Lactate (1%)	-ve (194)
Aztreonam	+ ve (682)
Resistance to:	
Rifamycine SV	- ve Sensitive (235)
Troleandomycin	- ve Sensitive (85)
Minocycline	- ve/+ve Sensitive (361)
Lincomycin	- ve /+ve Sensitive (364)
Niaproof 4	- ve Sensitive (73)
L-Histidine	- ve Sensitive (55)
Vancomycin	- ve /+ve Sensitive (407)

Table 4: Biochemical characteristics of isolate No.1

Test	Reaction of selected stain
Fatty acids pattern:	
γ-Amino-Butryric Acid	-ve (273)
-Hydroxy- Butryric Acid	-ve (34)
β-Hydroxy -D, L- Butryric Acid	-ve (119)
α - Keto- Butryric Acid	-ve (96)
Acetoacetic Acid	-ve (192)
Propionic Acid	+ve (111)
Acitic Acid	+ve (188)
Formic Acid	-ve (218)
β-Hydroxy -Phenylacetic Acid	-ve (62)
Methyl Pyruvate	-ve (431)
D-Lactic Acid Methyl Ester	-ve (234)
L- Lactic Acid	-ve (15)
Citric Acid	+ve (459)
α - Keto- Glutaric Acid	-ve (46)
D-Malic Acid	-ve (243)
Bromo-Succinic Acid	-ve (488)
Nalidixic Acid	+ve (603)
D-Galacturonic Acid	-ve (173)
L-Galactonic Acid Lactone	+ve (76)
D-Gluconic Acid	+ve (149)
D-Glucuronic Acid	+ve (192)
Glucuronamide	- ve/+ ve (27)
Mucic Acid	+ve (323)
Quince Acid	- ve/+ ve (4)
D-Saccharic Acid	-ve (26)
L-Aspartic Acid	+ve (138)
L-Glutamic Acid	+ve (154)
L-Pyroglutamic Acid	+ve (40)
Guanidine HCl	-ve (180)
Fusidic Acid	-ve (97)
N-Acetyl-Neuraminic	+ve (291)
D-Aspartic Acid	-ve (349)

Table 5: Utilization of different carbon and nitrogen sources by the isolate No. 1

Test	Reaction of selected stain
Growth on sole carbon sources (0.1 % w/v):	
α –D-Glucose	+ve (185)
D-Maltose	+ve (145)
D-Trehalose	+ve (94)
D-Cellobiose	+ve (155)
D-Mannitol	-ve (464)
Sucrose	+ve (79)
L-Rhamnose	+ve (55)
D-Rhafinnose	+ve (126)
D-Fructose	+ve (234)
D-Turanose	+ve (186)
Gentiobiose	+ ve (342)
Stachyose	- ve/+ ve(21)
α -D-Lactose	- ve/+ ve(2)
D-Melibiose	+ ve (83)
β-Methyl-D-Glucoside	+ ve (247)
N-Acetyl-D-Glucosamine	+ ve (231)
N-Acetyl-β-D-Mannosamine	+ ve (151)
N-Acetyl-D-Galactosamine	+ ve (125)
D-Mannose	- ve/+ ve (15)
D-Galactose	+ ve (136)
3-Methyl Glucose	+ ve (122)
D-Fucose	+ ve (114)
L-Fucose	+ ve (59)
D-Sorbitol	+ ve (56)
D-Arabitol	- ve (366)
Myo-Inositol	- ve/+ ve (2)
Glycerol	- ve/+ ve (7)
Glycyl-L-Proline	+ ve (31)
D-Glucose-6-PO4	+ ve (420)
No carbon sources	- ve (0)
Utilization of nitrogen sources:	
Potassium Tellurite	- ve (100)
L-Arginine	+ ve (148)
L-Alalnine	+ ve (92)
No nitrogen source	- ve (0)

(+): detected and (-): not detected.

4. DISCUSSION:

In this research, samples were collected from waste water to isolated bacteria that produced α-amylase enzyme, all conditions of α-amylase activity were studied. Water sample from Pharaonic Lake, Minoufia Governorate, Egypt, was collected according to standard methods for examination of water and waste water [15]. All thirty tested isolates were screened for their ability of α-amylase production, the organisms were inoculated on starch nitrate culture medium plates. contained [18], 324 (gl): soluble starch, 20.0 ; KNO ,1.0; K HPO ,0.5; -1 MgSO4 2 4 2 .7H O, 0.5 ; FeSO .7H O, 0.05; and agar, 20 ; sea water or lake water 1L. Incubation at 30°C was carried out for 24 hour after which the plates were 2 flooded with Gram's iodine solution (0.1% I and 1% KI) and the colonies with the largest halo-forming zone were chosen for further investigations [19].

Among the thirty isolates obtained No. 1; No. 2; and No. 3 were found to produce large clear zones than the other isolates indicating high α-amylase production. The isolate No.1 was chosen as the experimental organisms and was characterized in detail.

The α-amylase enzyme was produced by man y microorganisms like *B. licheniformis* [24], *B. subtilis* [25,26,27], *Aspergillus niger* 33-19 CNMN FD 02A [13]., *B. stearothermophilus* [28]. *A. oryzae* [29] and Strains of *B. stearothermophilus* and *B. amylolyticussecreted* [30].

Micro Analytical Center, Faculty of Science, Cairo MIRCEN, Faculty of Agriculture, ASU BiologDG (2013). Biolog Gp Data Base. Relase 15G Hayward, CA: Biolog. according to Berg's Mannual of systematic Bacteriology [23].

Isolate No. 1 was similar to *Alloiococcus otitis* in the spore chains were in coccus form, gram positive stain, characterized and identified, that the selected strain,

utilized varies carbon sources for growth as α –D-Glucose, D-Maltose, D-Trehalose, D-Cellobiose, Sucrose, L-Rhamnose, Rhafinnose , D-Fructose, D-Turanose, Gentiobiose, Stachyose, α -D-Lactose , D-Melibiose, β-Methyl-D-Glucoside, N-Acetyl-D-Glucosamine, N-Acetyl-β-D-Mannosamine, N-Acetyl-D-Galactosamine, D-Mannose, D-Galactose, 3-Methyl Glucose, D-Fucose, L-Fucose, D-Sorbitol, D-Arabitol, Glycerol, Glycyl-L-Proline,D-Glucose-6-PO4, but D-Mannitol and Myo-Inositol were not utilized. Also, it utilized various nitrogen sources for growth as L-Arginine and L-Alalnine but Potassium Tellurite was not utilized and Gelatin, Dextrin, D-Salicin, Inosine, L- Serine and Pectin were observed, Degradation tolerate up to (1 % -8%) NaCl. Culture are susceptible to Rifamycine SV, Troleandomycin and L-Histidine. Growth is best on Bennett at 30 °C. The fatty acid compositions of the isolate examined is shown in Tables (3, 4 and 5). The fatty acid profiles revealed that Nalidixic Acid (603), Citric Acid (459) were the predominant fatty acids.

Integration to this information that the isolate No. 1 was identified as *Alloiococcus otitis.* The present results indicated that Bennett medium was the best medium and the optimum incubation period for α-amylase production was after 8 hours. Previous studies demonstrated. The strain was identified as *M. sanguineus* and maintained on Potato Dextrose Agar (PDA) medium [12]. *Arthrobacter psychrolactophilus* ATCC 700733 grew with a doubling time of 1.5–2.3 h (22°C) and produced up to 0.2 units/mL (soluble starch assay) of extracellular amylase in tryptic soy broth without dextrose (TSBWD) containing 0.5% or 1.0% (w/v) soluble starch or maltose as the fermentable substrate [14].

The present results showed that the maltose was the best carbon sources for production of α-amylase by isolate No. 1. In this concern, [31]; Carbon sources such as galactose, glycogen and inulin have been reported as suitable substrates for the production of amylases by *B. licheniformis* and *Bacillus sp* I-3. Also; In this concern, [5,32, 33] suggested that, Starch and glycerol were known to increase enzyme production in *B. subtilis* IMG22, *Bacillus sp*. PS-7. And Bacillus *sp*. I-3. Soluble starch has been found as the best substrate for the production α-amylase by *B. stearothermophilus* [28*]*. *Bacillus sp.* was noted to give a maximum raw starch digesting amylase in a medium containing lactose (1 %) and yeast extract [34]. *Thermomyces lanuginosus* was reported to give maximum α-amylase yield when maltodextrin was supplemented to the medium [35]. It is well known that, among nitrogen sources urea was considered to be the best inducer for the highest α-amylase production by isolate No.1. While, yeast extract along with ammonium sulfate also gave significant enzyme productivity (110 %) by *A. oryzae* [29]. Addition of corn steep liquor and ammonium hydrogen phosphate to SSF involving Amaranthus grains as substrate by *A. flavus* gave high enzyme yield [36].

Hyperthermophilic archae such as *Pyrococcus furiosus, P. woesei* and *Thermococcus profundus* yielded optimuma-amylase at pH = 5.0 [37]. *Rhodothermus marinus* was reported to yield good enzyme levels at initial pH range 7.5 to 8.8 [8]. This was almost in the same harmony with the present investigation which reached that a maximum production of α-amylase at pH = 8.0 for all *isolates* (No.1) and who demonstrated that, Thermophilic anaerobic bacteria *Clostridium thermosulfurogenes* gave maximum titres α-amylase at pH = 7.0 [38].

Our results clarified that the purified fraction from isolate No.1 was proved by the appearance of a single band corresponding to 38 k Da in SDS-PAGE, and the enzyme was purified 67-fold with amylase units 225 ml and specific activity of 234.4 U/mg. In this concern [39], found that *Streptomyces* strain K_{1-02} which was identified as *Streptomyces albidoflavus*, its Alfa-amylase enzyme was purified to homogeneity b y a two- step procedure. This enzyme had a molecular weight of 18 K Da. Also, *Streptomyces caespiyosus* produced protease enzyme had a molecular weight of 14 k Da by SDS-PAGE [40].

In the present study, the purified enzyme was stable at all degrees of temperatures (20 –70 ºC). This demonstrated a good match between the experimental value and predicted value thus substantiating the proposed model. [12]. *A. psychrolactophilus* amylase exhibited maximum activity on soluble starch at 40–50 ºC in 3-h incubations using un-concentrated supernatant fractions of cultures. The optimum pH activity was observed between 7.5-8.5. Optimum time for production of bacterial population were 48hrs.

On the other hand, the pH affects the ionization of amino acids, which dictate the primary and secondary structure of enzyme and hence control its activity [41].

In Our Results, A typical bell-shaped curve is obtained with the maximum α-amylase activity at pH 9.0 for all isolate (No.1). On contrary [42], demonstrated that the acidic pH activity by *Bacillus cereus* was observed 6. And also for determination of suitable pH range for enzymatic activity by *Bacillus subtilis* (MTCC 121) Using Solid State Fermentation, pH enzyme assay buffers were varied as 6.6, 7.1, 7.6, and 8.6. Maximum enzyme activity was observed at pH 7.1 (8.74 µmol/mg/mL) at 40°C. The use of more alkaline buffer resulted in sharp decline of enzyme activity. [43].

The present investigation, showed that, starch was the best as substrate for the purified α-amylase produced by isolate (No.1). This result, in similar studies, [19,44] noticed that the highest enzyme activity by *Bacillus sp.* was attained at 2.0% starch, whereas the maximum activity of enzyme by Pseudomonas fluorescence was ranged from 1.5% to 2.5% starch and decreased at 3.5% starch which have been reported by [45].

Our results showed that butanol was the best solvent for the purified α-amylase produced by isolate (No. 1. These results show high stability of the α-amylase against denaturation or unfolding in the presence of solvents. This capability may be due to disulfide bonds, presence of the disulfide bond(s) in this α-amylase was found to be

essential for its activity. There are number of reports on importance of disulfide bonds for stability of the protein against solvents [46]. α-amylase is very useful for fermentation and reactions in the presence of solvents. One of the most important advantages of this property is to reduce or abolish microbial contamination during degradation reaction.

The present study, showed that, CaCl2, MgCl2 and KCl were the best activator for the purified α-amylase produced by isolate (No. 1. On the other hand, the other tested metallic ions gave a variable inhibition for α-amylase activity. This is in agreement with that most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca2 +, Mg2+, Mn2 + , Zn2 + , Fe 2 +etc ;[47]. Ca^{2+} was reported to increase a-amylase activity of an alkaliphilic Bacillus sp. ANT-6 [48]. The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure [32]. Ca-independent enzymes have also been reported.

Our results indicated that, there is a continues decreasing residual activity due to the increase of SDS and H_2O_2 concentration for enzyme produced by isolate (No.1). On the contrary Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability. Therefore, addition of these surfactants is used for the production of extracellular enzymes. Addition of Tween 80 (1.3 %) to the fermentation medium increased α-amylase production by 2-fold in *Thermomyces lanuginosus* [49]. A study on the effect of supplementation of polyethylene glycols (PEG) (molecular mass of 600, 3000, 4000, 8000 and 20 000) in fermentation medium for a-amylase production by two Bacillus sp. indicated that 5 % PEG 600 and PEG 3000 yielded 31 % increase in enzyme production by *B. amyloliquefaciens* and 21 % increase by *B. subtilis* [50, 51] compared the effect of various surfactants in the production media of Ca^{2+} independent a-amylase by *Geobacillus thermoleovorans*. Cholic acid, an anionic surfactant, and Tween 80, neutral surfactant, gave 2-fold enzyme yields at a concentration of 0.03% mass per volume ratio. A mixture of PEG 8000 and SDS showed an inhibitory effect on enzyme production. Surfactants such as SDS, cholic acid, Tweens, etc. were reported to increase cell permeability, thereby enhancing enzyme yield [51].

Amylases are used commercially for desiring of textile fabrics, in preparing starch coatings of paints, in removing wallpaper, sugar induction by the production of sugar syrups from starch which consists of glucose, maltose and higher oligosaccharides, pharmaceutical and in preparing cold water dispersible laundry starches. To meet the demands of these industries low-cost medium is required for the production of amylases [52]. Nowadays the potential of using microorganisms as a biological source of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms. Amylases can be obtained from several sources such as plant, animal and microbes such as bacteria and fungi [53].

The microbial source of amylases is preferred to other sources because of its plasticity and vast availability. Until now all commercial enzymes have been derived from cultivated bacteria or fungi. Bacterial amylases are generally preferred for starch processing. Among bacteria, *Bacillus species* such *as B. subtilis, B. stearothermophilus, B. macerans, B. megaterium and B. amyloliquefaciens* were the best producers of thermostable α-amylase using submerged fermentation and these have been widely used for commercial production of the enzyme for various applications [54, 55], *Clostridiumthermosacharolyticum, Cl.Thermohydrosulfuricm, and Pseudomonas sp.* [56].

Thus at least it can be said that Alfa-amylase enzyme has wide application in various industrial and medical fields and this will be the aim of future work.

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