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Effect of Environmental and Nutritional Conditions on Amylase Production by *Bacillus subtilis*

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ABSTRACT

The research was carried out for isolation of amylase producing bacteria from various starchy locations. Thirty one bacterial isolates were screened in order to select the best amylase producing and identified as *Bacillus subtilis*. Optimization environmental condition factors were studied as ;organic nitrogen sources (beef extract, peptone, yeast extract, urea, or inorganic as; ammonium chloride, ammonium sulfate and sodium nitrate).Also carbon sources (sucrose, anhydrous dextrose, D+ glucose, lactose, corn starch, maltose, soluble starch and potato starch) were studied. The highest yield of amylase production by *B. subtilis* were achieved in cultures supplemented with sodium nitrate was; 1.34±0.032^{c} U/ml/min. on the other hand, maltose gave a high yield of amylase production 0.511±0.046 U/ml/min as carbon source. The optimum pH was 9.0 which gave 1.49±0.055 U/ml/min, while temperature 30c^{o}was better which gave 0.544±0.015 U/ml/min. Maximum amylase production was obtained by application of cheaper agro-industrial wastes type as carbon source, the highest yield was obtained with banana peels which gave; 0.638±0.004 U/ml/min.

Keywords: Environmental factors conditions, agro-industrial wastes, fermentation technique, amylase production, *Bacillus subtilis*.

INTRODUCTION

Amylase play an important roles in the biotechnology industries with many potential applications as foods, fermentations, textiles , papers and pharmaceutical industries(Pandey *et al*., 2000; Sanghvi *et al*., 2011) .

Amylases can be obtained from several sources such as plants, animals and microorganisms. The microbial sources 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 easy of improving the enzyme productivity through various methods from this environmental factor optimization.

Optimization of culture conditions is very important for maximum microbial growth and enzyme production by microorganisms (Kathiresan & Manivannan 2006). Among these physical and chemical factors as; optimum temperature, pH value, carbon and nitrogen sources are the most important for enzyme production by microorganisms (Gupta *et al*. 2003).

This study aimed to isolation, identification and improvement amylase production by bacteria from different starchy locations, determination optimized environmental conditions were; physical, chemical and nutritional factors were achieved to increase amylase productivity.

MATERIALS AND METHODS

Samples collection

Samples were collected from various starchy locations as follow: four samples from Chips factory at Tanta city - Gharbia Governorate, two samples from Potato washing water first (W_1) and second (W_2) stage; water sample after slicing potatoes (PW) and sample of potato peels (PC)), tow sweeping dust sample from racket bleaching rice (R) and Pasta factory at Qulin city - Kafr El-Sheikh Governorate.

Isolation, purification and identification of amylolytic bacteria

Bacillus bacterial isolation was performed by serial dilution method, of 0.1 ml sample suspension serial diluted from 10^{-1} up to 10^{-6}, dilution was inoculated on starch nutrient agar medium. The bacterial colonies which having amylase producing ability by clear surrounding after flooding with iodine solution. Amylolytic colonies were picked up and purified by more streaking times and microscopically checked. Bacterial isolates were identified as *Bacillus subtilis* as characteristics; microscopic, cultural, biochemical and biolog system report.

Optimization of culture conditions

These experiments were sequential trials for determination the optimum conditions for amylase production by the most active bacteria, each experiment was carried out in triplicate.

Effect of different carbon sources

Different carbon sources as; sucrose, anhydrous dextrose, D+ glucose, lactose, corn starch, and maltose and potato starch were studied by replacing the carbon source (soluble starch) in the production media. All these sources were studied at 1% initial concentrations. The amylase production was determined after 48 h of incubation at 30 ºC.

Effect of different nitrogen sources

The effect of nitrogen sources on amylase production were investigated by supplement of production media (without nitrogen source) with 0.2% (Demissie 2014) organic nitrogen sources as; beef extract, peptone, urea, yeast extract and inorganic nitrogen sources as; ammonium chloride, ammonium sulphate as a control. The amylase production was determined after 48 h of incubation at 30 ºc.

Effect of metal salts

The effect of metal salts ions on amylase production was determined by adding different metal salts individually in the fermentation medium as;(KH_2PO_4 1g, $(NH_4)_2SO_4$ 2g, Na_2HPO_4 2.5g, soluble starch 10g, distilled water 1000ml and pH adjusted to 7.0) . The metal salts selected study are; $CaCl_2$, $MgSo_4$, $CuSo_4$ and NaCl at 0.1% concentration (Akcan *et al*., 2011). The amylase production was determined after 48 h of incubation at 30 ºC.

Effect of pH values

To determine the optimum pH for amylase production, fermentation medium was adjusted at pH varying as; 5.0, 6.0, 7.0, 8.0, and 9.0 under standard conditions, pH measurements were carried out with pH meter glass electrode.

Effect of incubation temperature

Temperature was studied by incubating the inoculated media at various temperatures as; 25, 30, 35,

40 and 45°C with control at 30 °C. The bacterial culture media containing secreted amylase was centrifuged at 5500 rpm for 30 min at 4°C to remove cells and the protein in the supernatant. Several ammonium sulfate at 40, 60 and 80% concentrations was added to 10 ml of clear culture supernatant. Then there were kept overnight at 4°C centrifuged at 5500 rpm for 30min at 4°C. The pellets and supernatant without ammonium sulfate were collected and dissolved those pellets in 5ml of 0.2M phosphate buffer pH 7.0, both of dissolved pellets and supernatant were dialyzed against distilled water using cellophane paper. All supernatant and pellets were checked for the enzyme activity.

Effect of agro-industrial wastes type

Amylase production was carried out in solid state fermentation (SSF) by using cheaper agro-substrates as; wheat bran, rice bran, potato peel and banana peel (dried at 60ºC and ground to powder form). 5g of each substrates weighed and add to 100 ml flasks containing 5 ml of basal salt solution medium. Each flasks was inoculated after sterilization with 1ml of bacterial inoculum then incubated at 30°C (Suganthi *et al*., 2011).After incubation period 4 days, 22ml of 0.1M phosphate buffer (pH 7) was added to each flask and shacked in shaker water bath at room temperature for 15 minutes at 120 rpm. The mixture was filtered through medical gauze and centrifuged at 5000 rpm at 4°C for 30 minutes. The filtrate was used as the crude enzyme amylase activity and assayed by dinitro salicylic acid method.

Amylolytic assay

Bacterial amylase was determined using dinitro salicylic acid method (Miller 1959). According to the method, the reaction mixture contained 1ml of 1% soluble starch dissolved in 0.02M phosphate buffer (pH 6.9) and 1ml enzyme extract (culture supernatant) was incubated at 37 °C for 30 min (Mukesh kumar *et al*., 2012)The reaction was stopped by adding 2ml dinitro salicylic acid (DNSA) solution and heated for 5 min in boiling water. After that, 1ml of Rochelle salt solution 40% to stabilize the color and cooling to room temperature in a cold water bath. The concentration of the reducing sugar was measured at 540 nm by colorimeter using glucose as standard. The blank contained of reaction mixture without enzyme extract. One unit of enzyme activity was defined as the amount of enzyme that formed 1 mg of reducing sugar as glucose per minute under the assay condition by applying the following formula (Sudha, 2012).

$$\text{Enzyme activity (IU/ml/min)} = \frac{\text{Amount of reducing sugar} \times 1000}{\text{Molecular weight of glucose} \times \text{incubation time}}$$

Amount of reducing sugar = Absorbance at 540 / Slope of glucose standard.

RESULTS AND DISCUSSION

Isolation, purification and screening of amylolytic bacteria

All bacterial isolated from starchy locations samples data presented in Table (1). Out of thirty one will purified and screened of amylolytic were observed TB1 isolate a high active as compared with other isolates.

Table 1. Amylolytic activity, halo and colony diameter (after 24 h) produced by selected bacterial isolates.

Isolate number	Isolation source	Clear zone (mm)	Growth diameter (mm)	Halo diameter to colony diameter ratio
B3	P.W	3	3.3	0.90
B4	P.W	3	7	0.42
B2	P.W	2.6	8	0.32
TB1	P.W	4.3	3	1.4
TB22	P.W	1	3.4	0.29
TB2	P.W	2.6	6	0.43
B22	P.C	1	4.6	0.21
B1	P.C	2	5	0.4
B5	P.C	3	6	0.5
B3	P.C	1	6	0.16
B6	P.C	2.3	6.6	0.34
B7	P.C	2.3	5	0.46
SN1	W1	4.3	6.6	0.65
B11	W1	1.5	4	0.37
B12	W1	2.3	3.6	0.63
SN10	W1	1.3	4	0.32
B5	W1	3	6	0.5
B10	W2	3.6	4	0.9
B13	W2	3	4	0.75
B14	W2	3	4.6	0.65
B8	R	2.6	6.3	0.41
B23	R	2.6	4	0.65
B29	R	3	6	0.5
B10	R	1.3	4	0.32
TB3	R	2.3	5	0.46
SNS	R	2.6	4	0.65
TB4	R	1.3	5	0.26
TB21	R	2.3	6	0.38
TB6	M	2.6	5	0.52
B17	M	2	4	0.5
B24	M	2.3	6.3	0.36

Where:W1 Potato washing water first stage sample , W2 Potato washing water second stage sample, PW water sample after slicing potatoes, PC sample of potato peels, R racket bleaching rice sample and M sweeping dust sample from Pasta sample.

Identification of the selected bacteria

The morphological and biochemical identification testes of the amylolytic bacterial isolate TB1 showed that the isolate is Gram positive rods, endo-spore forming, aerobic, urase positive, gelatin, starch and casein hydrolyzed, citrate utilized, catalase positive, indole not produced and good growth were detected at 30 and 40 (Table 2 and 3), this isolate may be belong to *Bacillus subtilis*. Biolog system results cleared that bacterial isolate TB1 related to *B.subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of amylase and these have been widely used for commercial production of the enzyme for various applications (Vihinen, and Mantasala 1989, and Pandey, *et al*., 2000).

Optimization of environmental conditions on amylase production by *B. subtilis*

In this respect, group sequential experiment to determine favorite environmental condition was carried out for analysis production.

Effect of carbone sources

Amylases are an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product (Morkeberg *et al*., 1995). The enzyme production has been

greatly affected by the addition of different carbon sources. The carbon sources affect not only the mode of amylase formation but also the rate with which carbohydrates are metabolized (Dubey *et al*., 2000). Supplementation of the bacterial fermentation media with different carbon sources was screened at the concentration of 1%. Results in Table (4) show that maltose gave the highest amylase production (0.511±0.046) U/ml/min followed by potato starch (0.365±0.001) U/ml/min respectively. Results are in agreement with those obtained by Bhattacharya *et al*. (2011) reported that maltose acted as the best carbon supplement possibly because of its interaction as an inducer with the repressing protein.

Table 2. Cultural and Microscopic characteristics of the selected isolated bacteria.

Characteristics	Results
Form	Irregular
Elevation	Flat
Margin	Lobate
Color	Pale
Surface	Non shiny
Diameter of colony after 24h	4mm
Optical form	Echinulate
Distribution and appearance	Sediment
Growth at	
20℃	*
30℃	**
40℃	**
50℃	*
Shape of vegetate cell	Rod
Gram staining	Positive
Spore formation	Positive

Table 3. Biochemical and cultural characteristics of the selected isolated bacteria.

Characteristics	Results
Aerobic growth	+
Anaerobic growth	-
Urase	+
Gelatin liquefaction	+
Starch hydrolysis	+
Casein hydrolysis	+
Citrate utilization	+
Catalase	+
Indole production	-

+ positive, – negative

Table 4. Effect of different carbon sources on amylase production by *B.subtilis*TB1.

Carbon source	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
Soluble starch (control)	0.893±0.042a	0.346±0.017a
Sucrose	0.418±0.061b	0.157±0.024b
Anhydrous Dextrose	0.708±0.010c	0.272±0.004c
D+ Glucose	0.640±0.000c	0.246±0.000c
Lactose	0.684±0.070c	0.263±0.027c
Corn starch	0.915±0.030d	0.355±0.011d
Maltose	1.30±0.011e	0.511±0.046e
Potato starch	0.940±0.000d	0.365±0.001d

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

Effect of nitrogen sources

The nitrogen sources are of secondary energy source to organisms, which play an important role in the growth and enzyme production. Different inorganic and organic nitrogen sources have influential on the ability of bacterial fermentation media with various nitrogen sources was tested and screened at the concentration of 0.2%. Results in Table (5) show that sodium nitrate was the best nitrogen source for amylase production (0.528±0.012) U/ml/min followed by organic nitrogen source yeast extract (0.340±0.031) U/ml/min. The results obtained are in concordance with those obtained by pervious investigators. Shaista *et al*. (2003) founded that maximum amylase activity upon supplement with 0.2% peptone as an inorganic nitrogen source for *Bacillus* species. Ramachandran *et al*. (2004) reported that ammonium salts enhanced the amylase production.

Table 5. Effect of nitrogen sources on amylase production by *Bacillus subtilis* TB1.

Nitrogen sources	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
Ammonium chloride	0.479±0.036a	0.180±0.014a
Urea	0.415±0.091b	0.156±0.036b
Beef extract	0.505±0.014a	0.192±0.005a
Sodium nitrate	1.34±0.032c	0.528±0.012c
Peptone	0.782±0.015d	0.302±0.003d
Yeast extract	0.879±0.078d	0.340±0.031d
Ammonium sulfate	0.841±0.029d	0.325±0.011d

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

Effect of metal salts

Effect of metal salts has been studied at initial concentration 0.1%. Results in Table (6) showed that the effect of various metal ions on amylase productivity by *B. subtilis* strains. The obtained results revealed that the metal ions affected the production of amylase and differ between the producer strains. Significant increase in enzyme production was obtained with $FeCl_3$ and $MgSo_4$ adding to bacterial fermentation medium of tested strains. The maximum amylase production by *B. subtilis* strain was found in media supplemented with $MgSo_4$ and amylase activity 0.454±0.003U/ml/min, whereas minimum amylases production was found in the media containing $FeCl_3$ with amylase activity was 0.075±0.004 U/ml/min. The results obtained are in line with those obtained by. Afifi *et al*. (2008) who found that metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme.

Table 6. Effect of metal salts ions on amylase production by *Bacillus subtilis*TB1.

Metal salts ions	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
Control	0.909±0.014a	0.342±0.023a
CaCl2	1.02±0.005b	0.398±0.002b
MgSo4	1.07±0.025c	0.454±0.003c
CuSo4	0.465±0.014d	0.176±0.005d
NaCl	0.698±0.019e	0.268±0.007e
FeCl3	0.212±0..009f	0.075±0.004

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

Effect of pH values

The fermentation medium pH is one of the important physical parameters which play an important role in enzyme production. The pH range observed during the growth of microorganisms also effect on product stability. pH is known to affect the synthesis and secretion of amylase just like its

stability. Results in Table (7) showed the effect of pH on the production of amylase by *B. subtilis* was observed at pH 9.0 (0.557± .000 U/ml / min). Obtained resultes are in agreement with those obtained by. Saleem and Ebrahim, (2013) who reported that the metabolic activities of microorganisms are very much responding to pH change. Deb *et al*. (2013) found that the maximum enzyme production by *Bacillus amyloliquefaciens* was obtained in a fermentation medium with initial pH 9.0.

Table 7. Effect of pH values on amylase production by *Bacillus subtilis*TB1.

pH values	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
5.0	0.471±0.017	0.18±0.003a
6.0	0.913±0.045	0.354±0.018b
7.0	1.04±0.011	0.409±0.003c
8.0	1.26±0.011	0.492±0.009d
9.0	1.49±0.055	0.589±0.022e

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

Effect of incubation temperature

The effect of varying incubation temperature on amylase production was studied to determine the optimum temperature for enzyme production.

Results in Table (8) showed that the optimum temperature for amylases production by *B.subtilis* was 30^0C. On other hand, the minimum amount of amylase production by *B.subtilis* was, 0.26±0.002U/ml / min and achieved at 45°C.

The obtained results are close with that obtained by Deb *et al*., (2013) who reported that the temperature was considered an important environmental factor which control the growth and production metabolic substances by microorganisms and this is usually varied from one organism to another. Saleem and Ebrahim, (2014) reported that the decrease in enzyme activity was observed at higher temperature because of change in membrane composition and cause protein catabolism.

Table 8. Effect of incubation temperature on amylase production by *Bacillus subtilis*TB1.

|Incubation temperature	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
25℃	0.915±0.035	0.354±0.014 a
30 ℃	1.38±0.04	0.544±0.015 b
35 ℃	1.18±0.005	0.465±0.003 c
40 ℃	0.925±0.013	0.370±0.003 a
45 ℃	0.663±0.017	0.26±0.002 d

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

Effect of agro-industrial wastes type

The ability of selected *B. subtilis* to amylase production by using agro-residues type under solid state fermentation (SSF) was studied. Results in Table (9) show that the banana peels gave the maximum enzyme production (0.638±0.004 U/ml / min) and found to be the best substrate for extracellular amylase production from *B. subtilis*, On other hand, the minimum amylase produced was, 0.312 ± 0.175 U/ml / min achieved using by potato peels. Obtained results are in agreement with those obtained by Jadhav *et al*. (2013) and Anaegbu it al. (2017) they reported that the increase production of amylase was achieved by the using of banana peels residues.

Table 9. Effect of agro-industrial wastes type on amylase production by *B. subtilis*TB1.

Agro-industrial wastes type	Reducing sugar concentration mg/ml	Enzyme activity U/ml/min
Control	1.22±0.003a	0.480±0.003a
Wheat bran	1.18±0.003b	0.462±0.002b
Rice bran	1.31±0.015c	0.515±0.006c
Potato peels	0.54±0.031d	0.312±0.175d
Banana peels	1.62±0.02e	0.638±0.004e

The mean difference is significant at the level of 0.05, ± indicates the standard deviation (SD) among the three parallel replicates in each column.

CONCLUSION

The objective of this study was to isolate some local amylolytic bacteria isolates from different starchy location and select a highest amylase producers. Optimization of amylase production by studying some physical, chemical and nutritional factors affecting on amylase production. The highest amylase production by *B. subtilis* TB1 was selected secretion it under suitable factors as; pH 9.0, incubation temperature 30^0C, magnesium sulfate, sodium nitrate, banana peels. Treatment of the selected *B. subtilis* TB1 by EMS lead to increase of amylase production of 29 isolates from parent and 52 isolates lowered from parent under submerged fermentation technique.

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تأثير الظروف البيئية والغذائية على إنتاج إنزيم الأميليز بواسطة بكتيريا *Bacillus subtilus*

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[١] كلية الزراعة جامعة الازهر بالقاهرة

[٢] كلية الاقتصاد المنزلى للبنات جامعة الازهر بطنطا

أجريت هذه الدراسة للحصول على بعض عزلات بكتريا الباسيلس المحلية ذات الكفاءة العالية فى إنتاج إنزيمات تحليل النشا (الأميليز)من مواقع يتوافر بها النشا والعمل على تحسين قدرة هذه البكتيريا عن طريق تحديد الظروف المثلى للنمو والحصول على أعلى إنتاجية من الإنزيم حيث وجد أن أفضل مصدر كربون لإنتاج الأميليز من عزلة *B. subtilis* كان باستخدام سكر المالتوز كمصدر للكربون بتركيز ١% حيث أعطت ٠.٥١١ ± ٠.٠٤٦وحدة إنزيم لكل مل فى الدقيقة . أفضل مصدر نيتروجين كان نترات الصوديوم حيث أعطت ٠.٥٢٨ ± ٠.٠١٢. أيضا أفضل تركيز النشا كان ٢% لبكتريا *B. subtilis* وأظهرت نتائج تأثير أملاح المعادن إلى زيادة انتاج سلالة *B.subtilis* للأميليز فى البيئة المحتوية على MgSo4.كما اوضحت النتائج أن زيادة انتاجية الإنزيمات المحللة للنشا من عزلة *B. subtilis* كانت أعلى عند pH ٩ ، وأشارت نتائج درجات الحرارة إلى زيادة انتاجية الأميليز عند التحضين على ٣٠ ْم حيث أعطت ٠.٥٤٤ ± ٠.٠١٥ وحدة إنزيم لكل مل فى الدقيقة. كذلك أوضحت نتائج استخدام المخلفات الزراعية الصناعية زيادة نشاط عزلة *B. Subtilis* فى إنتاج الإنزيمات المحللة للنشا فى البيئة المحتوية على قشر الموز المطحون بعد التجفيف حيث أعطت ٠.٦٣٨ ± ٠.٠٠٤ وحدة إنزيم لكل مل فى الدقيقة. .