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Evaluation of Amylase Activity Produced by Genetically Modified Bacillus Grown on Different Media Containing Sugar Crops Wastes

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ABSTRACT

In this investigation, α-amylase gene region from a thermophilic *Bacillus stearothermophilus* isolated from sugar beet juice obtained from Dakahlia Sugar factory (Egypt) was cloned to competent *Bacillus licheniformis* ATCC 27811 cells. The original and modified *Bacillus* strains were spread on different media composition and enzyme activity was determined. According to the results, the α-amylase enzyme was seen to have highest activity at media containing sugarcane bagasse and/or sugar beet pulp. The results revealed that modified *Bacillus* surpassed the original one at all studied media. This obtained result proofed that gene transferred into *BL* had effective action on increase α-amylase activity. The thermo-stability studies revealed that enzyme activity of modified bacillus was high for the first 8 hours at 90°C, it was ranged from 95-90 % after 6 hours (enzyme activity decreased from 7.80 to 7.02 gm glucose/ml) then, decreased to 65-63 % at the end of 24 hours.
Keywords: Amylases, *Bacillus*, Sugar Crops, Thermophilic enzymes, recombinant amylase gene.

INTRODUCTION

In any industrial biotechnological process, achieving high productivity is an essential factor for commercial success. Bacterial extracellular enzymes are an important class of industrial enzymes constituting approximately 20% of the enzyme market (Rao *et al.,* 1998 and Gupta *et al.,* 2003). Genetic improvement of bacterial extracellular enzyme production is achieved by applying a range of strategies based on molecular cloning tools. Amylases are very important enzymes that analyzed the starch and related polysaccharides.

Amylase used to obtain maltose, glucose and maltodextrins during industrial processes. Amylases are widely distributed enzymes in bacteria, fungi, higher plants and animals. Thermophilic enzymes are widely demanded in order to be stable at different process conditions.

α-Amylase has been used for starch liquefaction for decades, and the properties of the enzyme have been improved for industrial applications. Many commercial enzymes that are stable at a temperature of 95^{o}C or above have been developed by the enzyme industry from the α-amylase of *Bacillus licheniformis* (BLA).

Thermostable α-amylase isolated from *B. stearothermophilus* (BSTA) is widely used for starch hydrolysis in the food industry (Kirk *et al.,* 2002). Niu *et al.* (2009) constructed a new strain of *B. licheniformis* (CBBD302) carrying a recombinant plasmid pHY-amyL to increase α-amylase production. They found that production of BLA by *B. licheniformis* (CBBD302-pHY-amyL) is amongst the highest levels in Gram-positive bacteria reported so far. Rabbani *et al*. (2011) isolated and expressed the randomly mutated *α-amylase* gene from *B. subtilis* strain 168. The gene was ligated into expression vector pET-15b and then further confirmed using digestion analysis. They observed 3 clones with higher enzymatic activity compared with the wild type. Kühnel *et al.* (2011) showed that Sugar-beet pulp (SBP) consists of up to 75% w/w of carbohydrates (dry matter). Arabinose, glucose and galacturonic acid (GA) are the main sugar moieties present in complex polysaccharide structure.

A *B. subtilis* strain KCC103 was used to produce α-amylase in medium containing sugarcane bagasse hydrolysate (SBH). α-amylase production in SBH-medium was enhanced to 144.5 Um l (-1) (2.2-fold) by response surface methodology (Rajagopalan and Krishnan, 2008).

In this investigation, we aim to construct a *B. licheniformis* carrying a recombinant amylase gene from *B. stearothermophilus* to increase α-amylase production.

MATERIALS AND METHODS

1 Bacterial strain, growth conditions and amylase activity test

Sugar beet juice is exposed to high temperature (70-80°C) during processing and production of sugar (Sucrose) in sugar factories. A strain of bacteria was isolated from sugar beet juice obtained from Dakahlia Sugar factory (Dakahlia, Egypt). The strain was identified as *B. stearothermophilus* using API methods according to Logan and Berkely (1984). Two strains of *Bacillus* sp were used in this study *B. licheniformis* ATCC 27811 which was provided by VACSERA and *B. stearothermophilus* that described previously.

2 Effect of media containing sugar crop wastes on Bacillus strain

Nine media were used to spread *B.* strains. The composition of the media was presented in Table 1. The strains were spread on LB media containing sugar crops wastes with different levels and with or without starch.

Table 1. Cultured media codes and their composition:

No.	Codes	Media Composition
1	M1	LB broth + 1gm sugar beet pulp/100 mL
2	M2	LB broth + 1gm sugarcane bagasse/100 mL
3	M3	LB broth
4	M4	LB broth + 1gm starch/100 mL
5	M5	1 gm sugarbeet pulp +1gm starch/100 mL
6	M6	1 gm sugarcane bagasse + 1gm starch/100 mL
7	M7	1 gm sugarcane bagasse/100 mL
8	M8	1 gm sugarbeet pulp/100 mL
9	M9	1 gm sugarbeet pulp + 1 sugarcane bagasse/100 mL

The pH was adjusted to 7 with NaOH and medium was sterilized by autoclaving at 121°C and 1atm pressure for 15 min.

3 DNA Extraction, plasmids construction and cloning of amylase coding gene region

The genomic DNA of *B. stearothermophilus* carrying the target DNA was extracted using the CTAB-

method described by Doyle and Doyle (1987) and modified by Khaled and Esh (2008). The primers were designed based on the *B. stearothermophilus* α-amylase sequence (GenBank accession no. M57457.1). PCR mixture prepared and the reaction performed, then the product was run on 1 % agarose gel and visualized under the UV light. Agarose gel piece including the amplified gene region was extracted from gel and purified as described by Downey (2003). The amplified PCR product was digested using restriction enzymes and ligated into pET15b vector using the TA cloning kit vector (Invitrogen, USA). Competent cell of *E. coli* DH5α was prepared and transformation was carried out according to Tu *et al*. (2005). The cloning vector pET15b and pLip expression vector were digested with the NdeI and HindIII digestion enzymes as described in Burhanoğlu (2012). Then plasmid pLip with insert was transformed to competent B. licheniformis ATCC 27811 cell line, transformation was carried out according to Tu *et al*. (2005). The recombinant plasmid, designated as pLip-BSK1, was transformed in *B. licheniformis* ATCC 27811 and cultured in LB medium supplemented with kanamycin (80 µg/mL) for 32 h at 37oC.

4 Amylase activity assay

Amylase activity was determined by 3, 5-dinitrosalicylic acid (DNS)-based method for measuring the release of reducing sugar from soluble starch according to method of Miller (1959) and described by Shafaat *et al*. (2011). The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve.

5 Evaluation of thermal stability for enzyme

To evaluate of thermal stability for enzyme, 25 µl enzyme was incubated at 65 ºC, 80 ºC and 90 ºC for 24 hours (h) and 50 µl starch was added (Sajedi *et al*., 2005), then amylase assay was performed according to DNS method.

RESULTS AND DISCUSSION

1 Cloning of amylase coding gene region

Competent cell of *E. coli* DH5α was prepared and transformation was carried out. After incubation of the components cells on LB-amp agar plate contain starch, white colonies from the plate were chosen and plasmids were isolated from them. The recombinant plasmid, designated as pLip-BSK1, was transformed in *B. licheniformis* ATCC 27811 and cultured in LB medium supplemented with kanamycin (80 µg/mL) for 32 h at 37oC. The genetically modified *B. licheniformis* was extracted and conducted to amylase activity assay.

2 Amylase activity assay

Amylase activity was determined by DNS-based method for measuring the release of reducing sugar from soluble starch according to method of Miller (1959) and described by Shafaat *et al*. (2011). Table 2 and figures from 1 to 3 contain mean performance of bacterial strains cultured on different media calculated as gm glucose per mL.

Table 2. α-Amylase activity (gm glucose/mL) of *B. stearothermophilus* (*Bst*), original *B. licheniformis* (*BL*) and modified *B. licheniformis* (*BLm*) over three incubation periods (24, 48 and 72 h).

Media	24h				48h				72h			
Code*	*Bst*	*BL*	*BLm*	Mean	*Bst*	*BL*	*BLm*	Mean	*Bst*	*BL*	*BLm*	Mean
M1	8.61^{R}	10.13^{K}	12.30^{F}	10.35	10.09^{S}	8.58^{VW}	19.26^{A}	12.64	15.23^{H}	12.94^{M}	20.10^{B}	16.09
M2	4.29^{U}	5.05^{T}	12.36^{EF}	7.23	10.08^{ST}	8.57^{VW}	19.32^{A}	12.65	16.48^{F}	14.01^{K}	20.87^{A}	17.12
M3	8.88^{PQR}	10.45^{JK}	12.25^{FG}	10.53	12.98^{I}	11.03^{Q}	17.58^{B}	13.86	17.38^{CD}	14.77^{I}	20.10^{B}	17.42
M4	11.02^{I}	12.97^{E}	8.26^{S}	10.75	11.25^{OP}	9.56^{U}	13.44^{GH}	11.42	10.02^{QR}	8.51^{ST}	12.05^{N}	10.19
M5	8.53^{RS}	10.03^{L}	9.03^{P}	9.20	11.31^{O}	9.61^{U}	12.55^{J}	11.16	9.96^{R}	8.47^{T}	10.92^{P}	9.78
M6	13.71^{D}	16.13^{A}	8.91^{PQ}	12.91	12.24^{JKL}	10.40^{R}	12.10^{L}	11.58	10.18^{Q}	8.65^{S}	11.47^{O}	10.10
M7	9.98^{LM}	11.75^{G}	14.73^{C}	12.15	14.49^{E}	12.32^{JK}	15.69^{D}	14.17	17.14^{D}	14.57^{IJK}	14.92^{HI}	15.54
M8	9.27^{NOP}	10.91^{IJK}	15.38^{B}	11.85	14.08^{EFG}	11.97^{M}	15.32^{DE}	13.79	16.85^{E}	14.32^{JK}	16.19^{FG}	15.79
M9	9.53^{N}	11.20^{H}	15.24^{B}	11.99	13.90^{G}	11.81^{MN}	16.59^{C}	14.10	16.06^{G}	13.65^{L}	17.54^{C}	15.75
Mean	9.31	10.96	12.05	10.78	12.27	10.43	15.76	12.82	14.37	12.21	16.02	14.20

M1toM9 = code of media that presented in Table 1, Different letters refer to significant difference (P≤ 0.05) by Duncan's test

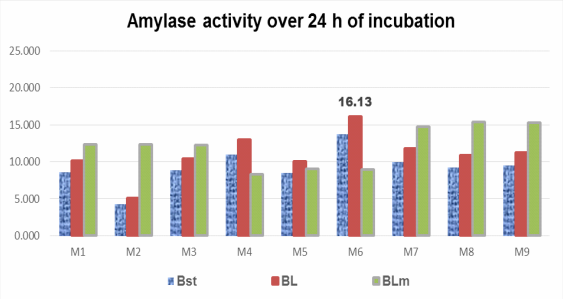

Figure 1. Amylase activity (gm glucose/mL) of *B. stearothermophilus* (*Bst*), original *B. licheniformis* (*BL*) and modified *B. licheniformis* (*BLm*) over 24 h.

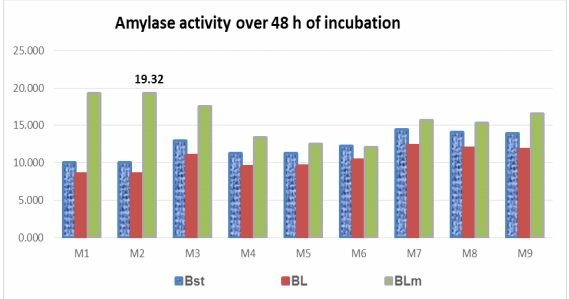

Figure 2. Amylase activity (gm glucose/mL) of *B. stearothermophilus* (*Bst*), original *B. licheniformis* (*BL*) and modified *B. licheniformis* (*BLm*) over 48 h.

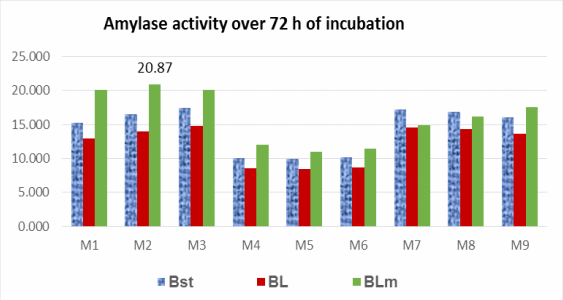

Figure 3. Amylase activity (gm glucose/mL) of *B. stearothermophilus* (*Bst*), original *B. licheniformis* (*BL*) and modified *B. licheniformis* (*BLm*) over 72 h.

These data showed the differences between the *B. licheniformis* ATC27811 (*BL*), modified *B. licheniformis* (*BLm*) and *B. stearothermophilus* (*Bst*) in their amylase activity calculated as gm glucose/ml. As shown in Table 2, it was observed that original *Bst* surpassed *BL* in their amylase activity (calculated as gm/glucose/mL) when they grow for 48 and 72 h, while *BL* was the superior after 24 h. The maximum α-amylase activity mean was recorded by *Bst* (14.366 gm glucose/ml) after 72 h (Table 2).

On the other hands, it was observed that modified *B. licheniformis* (*BLm*) surpassed *BL* in their amylase on all studied grown period (24, 48 and 72 h). The maximum α-amylase activity mean was recorded by *BLm* (16.02 gm glucose/ml) after 72 h.

3 Effect of media containing sugar crop wastes on amylase activity of *Bacillus* strain

Bacillus strains were spread on LB media contain sugar crops wastes with different levels and with or without starch; and amylase activity was measured.

Data presented in Table 2 revealed that best bacterial performance was observed with media containing sugarcane bagasse and/or sugarbeet pulp over three incubation periods. While enzyme activity recorded the highest value with M6 medium (sugarcane bagasse + starch) over 24 h of incubation (12.91 gm glucose/mL), M7 medium (sugarcane bagasse only) over 48 h of incubation (14.17 gm glucose/mL) and M2 medium (LB + sugarcane bagasse) over 72 h of incubation (17.12 gm glucose/mL). Amylase activity recorded the highest value on M2 and M6 media which contains sugarcane bagasse over the three incubation periods i.e enzyme activity recorded 16.13 gm glucose/ml with M6 over 24 h, 19.32 gm glucose/ml with M2 over 48 hrs and 20.87 gm glucose/ml with M2 over 72 h. The maximum amylase activity was recorded by *BLm* (20.87 gm glucose/ml) after 72 h of incubation at LB broth + sugarcane bagasse media (M2), however the minimum amylase activity by *BLm* was (8.26 gm glucose/ml) after 24 h of incubation at LB broth + starch media (M4). The results revealed that *BLm* was surpassed *BL* at most studied media.

4 Thermal stability for enzyme

Stability of the enzyme at different temperatures was determined in different time intervals. According to results presented in Figure 4, enzyme activity has relative stability for the first 8 hours over different temperature. Enzyme activity recorded values ranged between 7.55-7.021 gm glucose/ml at 90°C, while it ranged between 7.7-7.052 gm glucose/ml at 80 °C. On the other hands it ranged between 7.8-7.12 gm glucose/ml at 65 °C. The reduction percentage in enzyme activity was 7% at 90 °C, 8.4% at 80 °C and 8.7 at 65 °C after 8 h. Then, enzyme activity decreased to 5.02, 5.02 and 5.11 gm glucose/ml at 90, 80 and 60 °C respectively after 24 h. (Figure 4). Kolcuoğlu *et al*. (2010) reported that incubation at 80°C resulted about 80% enzyme activity after 72 h. For α-amylase of Bacillus sp., the activity was completely stable at 80°C for at least 90 minutes (Ben Ali *et al*., 1999). This obtained result proof that gene transferred into BL had effective action on increase amylase activity.

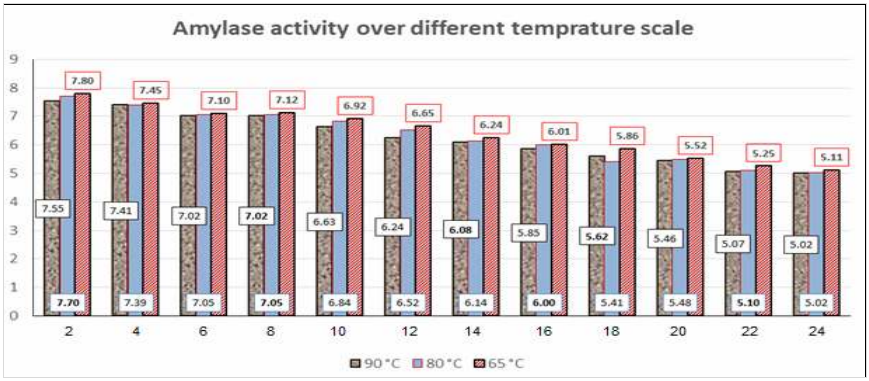

Figure 4. Amylase activity (gm glucose/mL) of modified *B. licheniformis* (*BLm*) incubated at 65, 80 and 90°C over 24 h.

CONCLUSION

α-Amylase gene region from a thermophilic *B. stearothermophilus* was cloned to competent *B. licheniformis* ATCC 27811 cells. The original and modified *Bacillus* strains were spread on different media composition and enzyme activity was determined. α-amylase enzyme was seen to have highest activity at media containing sugarcane bagasse and/or sugarbeet pulp. Enzyme activity recorded the highest value with M6 medium (sugarcane bagasse + starch) over 24 h of incubation (12.91 gm glucose/mL), M7 medium (sugarcane bagasse only) over 48 h of incubation (14.17 gm glucose/mL) and M2 medium (LB + sugarcane bagasse) over 72 h of incubation (17.12 gm glucose/mL). Amylase activity recorded the highest value on M2 and M6 media which contains sugarcane bagasse over the three incubation periods i.e enzyme activity recorded 16.13 gm glucose/ml with M6 over 24 h, 19.32 gm glucose/ml with M2 over 48 h and 20.87 gm glucose/ml with M2 over 72 h. The thermo-stability studies revealed that enzyme activity of modified *Bacillus* was high for the

first 8 h at 90°C, it was ranged from 95-90 % after 6 h (enzyme activity decreased from 7.80 to 7.02 gm glucose/ml) then, decreased to 65-63 % at the end of 24 h.

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تقييم نشاط إنزيمات الأميليز المنتجة بواسطة بكتيريا باسيلس محورة وراثياً فى بيئات مختلفة من مخلفات المحاصيل السكرية

خالد عدلى محمد خالد[1] ، كوثر سعد قش[2] ، محمد حسن عبد العزيز[2] و أمنية علاء إبراهيم بدر[3]

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إنزيمات الأميليز هي مجموعة من الإنزيمات الهامة جدا التي تحلل النشا والسكريات العديدة وهي تستخدم في العمليات الصناعية للحصول على المالتوز والجلوكوز وعديد الديكسترين وتوجد على نطاق واسع في البكتيريا والفطريات والنباتات والحيوانات. ويزداد الطلب على الإنزيمات المتحملة للحرارة حيث تكون مستقرة في ظروف عملية مختلفة. في هذه الدراسة تم عزل المنطقة الجينية المسئولة عن إفراز إنزيم ألفا أميليز من بكتيريا *Bacillus stearothermophilus* والتي تتواجد فى عصير بنجر السكر وتتحمل درجات حرارة عالية أثناء التصنيع بمصنع سكر الدقهلية. تم نقل الجين السابق الحصول عليه إلى خلايا بكتيريا *Bacillus licheniformis* ATCC 27811 التي تنتج إنزيم الأميليز بصورة كبيرة. وقد تم إنماء سلالات البكتيريا الأصلية والمعدلة على بيئات مختلفة وتحديد نشاط الإنزيم. وقد أظهرت النتائج أن إنزيم الألفا أميليز قد أعطى أعلى نشاط في البيئات التي تحتوي على باجاس قصب السكر أو لب بنجر السكر أو كليهما كما أظهرت النتائج أن البكتيريا المعدلة وراثياً قد تفوقت على البكتيريا غير المعدلة في كل البيئات تحت الدراسة. وهذه النتائج المتحصل عليها أعطت دليلا على أن الجين المنقول إلى *Bacillus licheniformis* قد أدى دوراً فاعلاً في زيادة النشاط الإنزيمى للألفا أميليز. كما دلت النتائج على أن نشاط الإنزيم للبكتيريا المعدلة كان مرتفعا لمدة 8 ساعات متصلة عند درجة حرارة 90 درجة مئوية، وقد تراوحت كفاءة الإنزيم بين 90-95% بعد 6 ساعات (حيث انخفض نشاط الإنزيم من 7.80 إلى 7.02 جم جلوكوز / مل) ثم انخفضت كفاءة الإنزيم إلى 63-65% في نهاية الـ 24 ساعة.