

Genetic Transformation of *Bacillus licheniformis* by Gene Responsible for α -Amylase Production in Media Contains Sugar Crops Wastes

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ABSTRACT

The importance of amylase enzymes is attributed to their ability to catalyze hydrolysis of starch and polysaccharides into maltose, glucose and/or maltodextrin for industrial processes. Their thermophilic characteristics are necessary to maintain stability under different conditions. Amylases are produced by, fungi, higher plants and animals. In the present study, thermophilic *Bacillus* (*Bacillus stearothermophilus*) isolated from beet pulp during sugar processing was used for cloning α -amylase gene into *Bacillus licheniformis*. The cloned and sequenced gene was 1827 bp in length. It has been registered in the GenBank (accession no. LC259133.1). This thermophilic amylase, with a molecular weight of 60 kD, was expressed and purified from the recombinant strain and matched to the α -amylase family protein of *Bacillus stearothermophilus* (GenBank accession no. M57457.1) using the NCBI database. The amino acid sequence of the cloned recombinant enzyme was 98% similar to that of the *Bacillus stearothermophilus* enzyme in the database. *Bacillus* strains were cultured and the activity of the enzyme was determined. The purified amylase on sugarcane bagasse and/or sugarbeet pulp media was highly active. Moreover, the modified *B. licheniformis* surpassed the other strain in cell mass amount and amylase activity on all media. These results confirmed that the gene transferred into *B. licheniformis* effectively increased amylase activity in this bacterium, while sugarcane wastes increased cell mass amount and enzyme activity. Also, thermostability of α -amylase was not clearly different between modified and original *B. licheniformis*. These provided evidence that gene transfer to *B. licheniformis* effectively increased its amylase activity but did not affect its thermostability. The enzyme produced in this study have high thermostability even 90 °C, high thermostability range allows it to be utilized in industrial applications, because their high yield, as well as their time and cost saving.

Keywords: Amylase; *Bacillus*; Sugar Crops wastes; Sugarcane; Sugarbeet, Cloning, thermostability

INTRODUCTION

For commercial success in biotechnological process, the most important factor is achieving high productivity. Enzymes from Bacteria are important in industry, constituting roughly 20% of the market (Rao *et al.*, 1998 and Gupta *et al.*, 2003). For decades, α -amylase used for starch decomposition, and their properties have been improved for industrial goals. Many commercial enzymes developed from the α -amylase of *Bacillus licheniformis* (BLA) have thermostability at high temperature. Thermostable α -amylases isolated from *Bacillus stearothermophilus* (BSTA) are also used for starch decomposition (Kirk *et al.*, 2002). Moreover, the *Bacillus licheniformis* (CBBD302) strain that carried the plasmid (pHY-amyL) was constructed to increase level of α -amylase production to be among the highest Gram-positive bacteria reported till now (Niu *et al.*, 2009). In addition, a mutated α -amylase gene (from *Bacillus subtilis* strain 168) was isolated and ligated into the expression vector pET-15b, three clones with high enzyme activity were observed (Rabbani *et al.*, 2011). The complex polysaccharide of sugar-beet pulp contains glucose, Arabinose and galacturonic acid and comprises up to 75% w/w carbohydrates on the dry matter (Kühnel *et al.* 2011). Sugarcane bagasse has been used to produce amylase from *Bacillus subtilis* (strain KCC103) which was enhanced to 2.2-fold (Rajagopalan and Krishnan, 2008).

The aim of the present study was to increase amylase production through genetic transformation by generating *B. licheniformis* strain carry a recombinant gene from *B. stearothermophilus* in order to increase amylase production.

MATERIALS AND METHODS

1-Bacterial Strains

This investigation was carried out in Sugar Crops Research Institute (SCRI), Agricultural Research Centre (ARC), Egypt. Processing of Sugar beet for sugar production need to expose the juice to high temperatures (70-80°C). In the present study, thermophilic *Bacillus* (*Bacillus*

stearothermophilus) was isolated from beet pulp during sugar processing in Daqahlia Sugar & Refining Company (Daqahlia, Egypt) and Delta Sugar Company (Kafr El Sheikh, Egypt); and tested using API methods described by Logan and Berkely (1984). Another *Bacillus* strains (*Bacillus licheniformis* ATCC 27811) provided by Egyptian Organization for Vaccines Services (VACSERA) were used.

2- Culture media

Both strains i.e. *Bacillus stearothermophilus* and *Bacillus licheniformis* were cultured on six different media with or without Luria-Bertani broth (LB broth), starch, sugar beet pulp, and sugarcane bagasse (Table 1).

Table 1. Culture media

No.	Codes	Media Composition
1	M1	LB broth
2	M2	LB broth + 1 % starch
3	M3	LB broth + 1 % sugar beet pulp
4	M4	LB broth + 1 % sugarcane bagasse
5	M5	LB + 1 % sugar beet pulp + 1 % sugarcane bagasse
6	M6	1 % sugar beet pulp + 1 % sugarcane bagasse

LB broth pre-sterilized by autoclaving at 121°C at 1 atm /15 min.

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

3-DNA Isolation, primer design and DNA amplification

Genomic DNA was extracted from *Bacillus stearothermophilus* using the modified CTAB-method (Khaled and Esh 2008). Target gene encoding α -amylase sequence was amplified using *BamyS* primer pairs. The primers pairs and restriction site were:

BamySF (Nde I, 5'-CATATGTTTTCACCGTTTGATGATTGTAC-3')

BamySR (Hind III, 5'-AAAGCAAATATACTGTACACGGCATTTCGAA-3') (restriction site underlined)

These primers were designed based on the GenBank accession no. M57457.1 (*Bacillus stearothermophilus* α -amylase gene).

The PCR product was run on a 1% agarose gel and visualized under UV light. The corresponding band to the amplified sequence was extracted and purified from the gel according to Downey, 2003.

4-Cloning of α -amylase Coding Region

Restriction enzymes (*Nde* I and *Hind* III) used for PCR product digestion, then ligated into the TA cloning kit vector (Invitrogen, USA). The Ligation was performed in a 20 μ l reaction mixture [2.0 μ l of Ligation Buffer + 4 μ l of T4 DNA Ligase + 1 μ l of the pET15b vector (provided by the Genetics and Breeding Dept., SCRI, ARC) + 12 μ l of the PCR product (20.8 ng/ μ l) + 1 μ l ultrapure water], that incubated overnight at room temperature. *E. coli* DH5 α competent cells were prepared then transformation was performed according to Zhiming *et al.* (2005).

5-Plasmid Isolation

The pETb15 vector containing the insert was isolated using a Mini Prep Plasmid kit and the amount of plasmid determined spectrophotometry at 260 nm. The cloning vector pETb15 was digested according to Burhanoğlu (2012) using the *Nde*I and *Hind*III enzymes and insert subsequently extracted from the gel. The same enzymes used to digest pLip expression vector also. The ligation reaction conditions were designed according to a vector-to-insert ratio of 1/10 in a reaction containing 5 x ligation buffers (4 μ l), T4 ligase (1 μ l), insert (9 μ l) and pFL (6 μ l) and incubated at room temperature overnight.

6-Competent *Bacillus licheniformis* Transformation

For expression the target gene encoding the α -amylase of *Bacillus stearothermophilus* (hereafter known as BSK1), the pLip plasmid containing the insert transformed into *Bacillus licheniformis* ATCC 27811 competent cells according to Zhiming *et al.* (2005). The pLip-BSK1 (recombinant plasmid) extracted and sequenced by the GATC Company using an ABI sequencer with appropriate primers.

7-Expressed protein and SDS-PAGE

Transformed *Bacillus licheniformis* ATCC 27811 containing pLip-BSK1 was cultured in kanamycin supplemented LB medium (80 μ g/mL) for 32 h at 37°C. Then cells subsequently recovered using centrifuge (10,000 \times g/ 4°C). CaCl₂ (5 mM) added to the supernatant and incubated at 75°C/ 40 min. Proteins removed using centrifuge (10,000 \times g/ 4°C), then supernatant collected and fractionated by ammonium sulfate 70%. Proteins recovered using centrifuge (8,000 \times g/ 4°C) and dissolved in 25 mM Tris-HCl buffer (pH 7.5) and diluted as 1 to 100,000 \times enzyme solution v/v, then a diethylaminoethyl (DEAE)-cellulose column (Sigma-Aldrich, St. Louis, MO, USA) used to remove pigments. SDS-PAGE gel was used to screen purified solution to detect purity and molecular weight of enzyme. Reaction carried out on 10 ml of 12% monomer separating gel and 5 ml of 4% stacking gel. Subsequently, the gel was incubated in 20% TCA (trichloroacetic acid) for 30 minutes with gentle shaking. Then, the gel was incubated in 0.05% Coomassie Brilliant blue R250 for 30 minutes on a shaker. Next, the gel was detained and monitored under white light with a camera.

8-Activity assay of Amylase

3,5-Dinitrosalicylic Acid method (Miller, 1959) was used to determine amylase activity by measuring the reducing sugar released during starch decomposition, then absorbance was measured at 540 nm, and standard curve generated using D-glucose (Shafaat *et al.*, 2011).

9-Temperature Stability

For temperature stability, 25 μ l of the enzyme incubated at 65°C, 80°C or 90°C for 24, 48 or 72 hours. Then, 50 μ l of substrate was added, and the amylase assay was performed.

10-Cell Mass (g/l)

After incubation completed, samples (50 mL) was taken from each two lines and centrifuged at 8000 rpm/15min. The cell pellets were collected and lyophilized. Dry weight was calculated and expressed in grams per liter (g/L).

RESULTS

1-Isolation of Genomic DNA and PCR Amplification

DNA isolated from *Bacillus stearothermophilus* and α -amylase sequence amplified in the existence of the primers that designated. Subsequently, agarose was used to run the product and photographed. We found that molecular weight about 1850 bp (Fig. 1).



Fig. 1. Gel electrophoresis of the target sequence (α -amylase) fragment using amylase-specific primers, M=DNA ladder and S=sample of target sequence.

2-Cloning of α -amylase coding sequence

α -Amylase gene sequence isolated from gel, ligated into the pET15b vector and inserted into *E. coli* DH5 α competent cells, then incubated on LB-amp agar plates. White colonies were selected from the plates and the plasmids isolated. The plasmid DNA was sequenced and compared, and the complete sequence of the insert was determined. The length of the target gene cloned into the pET15b plasmid was 1827 bp (Fig. 2).

This obtained sequence was compared with the reference gene sequence (GenBank accession no. M57457.1), and the alignment between the reference *Bacillus stearothermophilus* α -amylase complete cds gene (GenBank accession no. M57457.1). 99% of similarity between our gene sequences and M57457.1 GenBank accession was found (Fig. 3). Additionally, the constructed sequence of protein was calculated and as molecular weight and it was about 61.963 kD. Moreover, protein sequence matched the references enzyme by 95%. A comparison of protein sequences and the reference enzyme presented in Fig. 4.

The α -amylase gene isolated and sequenced, then ligated with the pLip expression vector (pLip-BSK1 construct shown in Fig. 5), followed by transformation into competent *B. licheniformis* ATCC 27811 cells.

3-Expression α -amylase gene into *Bacillus licheniformis*

To express gene encoding α -amylase enzyme of *Bacillus stearothermophilus* (thereafter known as BSK1), the pLip containing BSK1 (pLip-BSK1) was inserted into *Bacillus licheniformis* ATCC 27811 competent cell line, which was then cultured in kanamycin supplemented LB medium (80 μ g/mL) for 32 hrs/37°C. The enzyme was subsequently isolated from media and SDS-PAGE gel was used to determine molecular weight; the protein was approximately 60 kD (Fig. 6).

>*Bacillus* amylase gene
 cggtccgcccgcacgggtgaaagtcaccaatgtgggacgctcgtgatggcagaagatcaaaataaaattataatagac
 gtaaccgttcgaggttttcttcctgtttactctgtaacatttgatgaaggggaaagggcattgtgctaacgtttcaccgcatca
 ttcgaaaagatggatgttccgctcgcgctttttgctcaactcgtcgtgtctgcccaccggacagcccgcaagcgtgcc
 gaccgttttaaccggccaccatgagcagttttgaaatggtacttccgctccaccctactcggcgttgnitggcagctatggc
 cnaagggcccaatgnaagccaaasellalecggcelggcclecepetelltggelgecegecegelinagggaaeage
 cgaaggaagcgtgggttaaggatatacagactttatgaaotgggtgaattcaatcaaaaagggcgtccgcaaaaata
 gaaacaaaagcicaatctcfaagccattcaagcccccacgcccctggaatgcaaggttacgccaatgtcgtattcgcac
 staaagggccgcccggccgcaaggaatggatggcccccgaagcgaatccgicggacccgcaacccnagcnaatctcggg
 catctatcaaatccnagcctggacgaantttgattttccggccggggcaacccctactccagctttaggtggcctggaacc
 alltlatggcgttgalggcggnaagccgcaatllggccggcaatllacnaatllccggccggccggccggccggccggcc
 ggaagttagccagggaaacggaaactatgactacttaagtatgcccagccttgataggaatccggaaagcgtggaatgag
 ctanaagcgtgggcaaatgtatgtcaacacaggaacacttgatgggtccggcttggatccgctcaagcattatgaatgacg
 flllllctcgalggllgctgtatgpcgticcgacggcagccgcaatllccggllggggatallgggctatgcaatle
 acaagtgcacaattacattatgaaacaaacggagatcaccggacgatgcttggttgatgcccggttacacaacaatttt
 ataccgttcaaatcagggggcaatttgatagcgcagttaatgaccaactctctatgaaagatcaaccaacattggcc
 gtcaccttctgataatcatgacacggaaacccggccaagcctcagctcagctatggctgaccatgggttcaaacctgtggctt
 acgctttattctaaactcggcaggaaggataccgctgcttttattggtgactattatgcatccacaataataacattcctc
 gctgaaaagcaaatcgatccgctcctcatcgccgagggattatgcttacggaacgcaacatgattatcttgatcaccgga
 acatcaccgggtggacaagggaaagggtcaactgaaaaaacagatccggactggccgctgattgacacggatgacccgga
 ggaagcaaatggatgacgttggcaaacacacgcccgaagagttctatgacctaccggcaacccggagtgacacccgctc
 acatcaaacagtgatggatgggggaattcaagatcaatggcgggtcgggttccggttccggttccatgaaaaaacgctc
 tactatcgttggctgatcaaacaccgacgctggactgatattcgtccgttggaccgaaacacgggttggggcatggcctt
 gatgctctca

Fig. 2. Complete sequence of target region responsible for α -amylase gene

Score	Expect	Identities	Gaps	Strand
3003 bits (1626)	0.0	(686/1710(99%))	24/1710(1%)	Plus/Plus
Query 118	118	173		
Sbjct 285	285	304		
Query 174	174	233		
Sbjct 265	265	324		
Query 234	234	293		
Sbjct 325	325	384		
Query 294	294	353		
Sbjct 385	385	429		
Query 354	354	413		
Sbjct 430	430	489		
Query 414	414	473		
Sbjct 490	490	549		
Query 474	474	533		
Sbjct 550	550	609		
Query 534	534	593		
Sbjct 610	610	669		
Query 594	594	653		
Sbjct 670	670	729		
Query 654	654	713		
Sbjct 730	730	789		
Query 714	714	773		
Sbjct 790	790	849		
Query 774	774	833		
Sbjct 850	850	909		
Query 834	834	893		
Sbjct 910	910	969		
Query 894	894	953		
Sbjct 970	970	1029		
Query 954	954	1013		
Sbjct 1030	1030	1089		
Query 1014	1014	1073		
Sbjct 1090	1090	1149		
Query 1074	1074	1133		
Sbjct 1150	1150	1200		
Query 1134	1134	1193		
Sbjct 1201	1201	1260		
Query 1194	1194	1253		
Sbjct 1261	1261	1320		
Query 1254	1254	1313		
Sbjct 1321	1321	1380		
Query 1314	1314	1373		
Sbjct 1381	1381	1440		
Query 1374	1374	1433		
Sbjct 1441	1441	1500		
Query 1434	1434	1493		
Sbjct 1501	1501	1560		
Query 1494	1494	1553		
Sbjct 1561	1561	1620		
Query 1554	1554	1613		
Sbjct 1621	1621	1680		
Query 1614	1614	1673		
Sbjct 1681	1681	1740		
Query 1674	1674	1733		
Sbjct 1741	1741	1800		
Query 1734	1734	1793		
Sbjct 1801	1801	1860		
Query 1754	1754	1813		
Sbjct 1801	1801	1860		
Score	Expect	Identities <td>Gaps <td>Strand </td></td>	Gaps <td>Strand </td>	Strand
196 bits (106)	1e-45	106/106(100%)	0/106(0%)	Plus/Plus
Query 9	9	68		
Sbjct 52	52	111		
Query 69	69	114		
Sbjct 112	112	157		

Fig. 3. Nucleotide sequence comparison of references gene *B. stearothersophilus* α -amylase gene, complete cds (GenBank accession no. M57457.1) and target gene

Score	Expect	Method	Identities	Positives	Gaps
1097 bits(2838)	0.0	Compositional matrix adjust.	537/545(99%)	537/545(98%)	8/545(1%)
Query 13	MFLLAFLLTALLFCPTGQPAKAAAPFNGTHMQYFEWYLP----		DDGTLWTKVANEANNL		67
Sbjct 1	MFLLAFLLTALLFCPTGQPAKAAAPFNGTHMQYFEWYLP		DDGTLWTKVANEANNL		68
Query 68	SSLGITALLWLPAYKGTSRSDVGVGYDLYDLGDFNKGAVRTKYGT		KAQYLQAIQAAHA		127
Sbjct 61	SSLGITALLWLPAYKGTSRSDVGVGYDLYDLGDFNKGAVRTKYGT		KAQYLQAIQAAHA		120
Query 128	AGNQVYADVVDHKGADGTEHVDAVEVNPSDRNQEIISGIYQIAWTK		FDFFPGRNTYSS		187
Sbjct 121	AGNQVYADVVDHKGADGTEHVDAVEVNPSDRNQEIISGIYQIAWTK		FDFFPGRNTYSS		180
Query 188	FKWRVYHFDGVDNDESRKLSRIYKFRGIGKALDNEVDTEHNYDYL		HYADLDMDHPEVVT		247
Sbjct 181	FKWRVYHFDGVDNDESRKLSRIYKFRGIGKALDNEVDTEHNYDYL		HYADLDMDHPEVVT		240
Query 248	ELKAGAKHYVWTRTLDGFRDVAVKHKFSFFPDWLSYVRSQTGKPL		FTVGEYKSYDINKL		307
Sbjct 241	ELKAGAKHYVWTRTLDGFRDVAVKHKFSFFPDWLSYVRSQTGKPL		FTVGEYKSYDINKL		300
Query 308	HNVIKNTNG---TNSLFDAPLHNKPYTASKSGGTFDMRTLMTNTL		MKDQPTLAVTFVDNH		364
Sbjct 301	HNVIKNTNGDHPNTNSLFDAPLHNKPYTASKSGGTFDMRTLMTNTL		MKDQPTLAVTFVDNH		360
Query 345	DTEPGQALQSWDPWFKPLAYAFILTRQEGYPCVFYGDYGIPOVNI		PELKEKIDPLLIA		424
Sbjct 361	DTEPGQALQSWDPWFKPLAYAFILTRQEGYPCVFYGDYGIPOVNI		PELKEKIDPLLIA		420
Query 425	RRDYAYGTQHDYLDHSDIIGWTRREGVTEKPGSGLAALITDGP		GGSKIMYVGGKQAGKVFY		484
Sbjct 421	RRDYAYGTQHDYLDHSDIIGWTRREGVTEKPGSGLAALITDGP		GGSKIMYVGGKQAGKVFY		480
Query 485	DLTGNRSDTYTINSOGWGEFKVNGGSVSWVPRKTTVSTIAWSIT		TRPHTEFVRHTEPR		544
Sbjct 481	DLTGNRSDTYTINSOGWGEFKVNGGSVSWVPRKTTVSTIAWSIT		TRPHTEFVRHTEPR		540
Query 545	LVAMP	549			
Sbjct 541	LVAMP	545			

Fig. 4. Protein sequence comparison of target protein and references enzyme

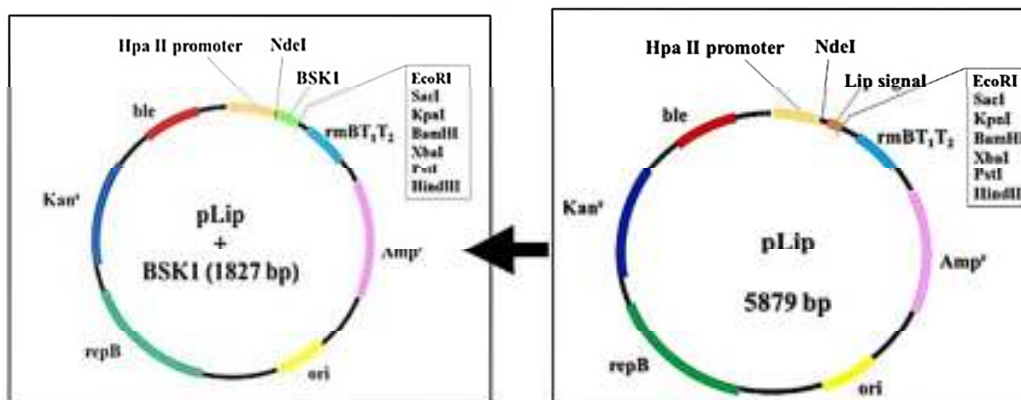


Fig. 5. pLip-BSK1 construct contains pLip plasmid + insert (amylase target gene sequence)

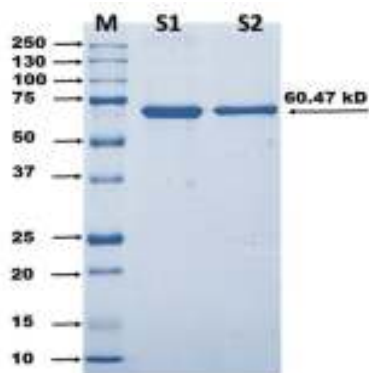


Fig. 6. SDS-PAGE of purified expressed protein, M= protein marker, S1 and S2= two identical enzyme samples

The protein solution concentrated at 40°C in a vacuum oven to 10 ml, measured with a NanoDrop and determined to be 4 mg/ml. Also, the protein concentration measured using Bradford method. Standard curve constructed and measured at 595 nm, the concentration was 1.1302 mg/ml. Thus, we obtained a total of 11.311 mg of protein.

4-Assay of α-amylase activity

α-Amylase activity by measuring reducing sugar released during starch decomposition was determined, the information regarding bacteria inoculated on different media (units of enzyme/ml) were presented in Fig. 7.

Data presented by Fig. 7 can be used to compare the differences between the amylase activity of *Bacillus licheniformis* (BL) and modified *Bacillus licheniformis* (BLm) in terms of enzyme activity (units/ml). It is revealed that the amylase activity of BLm surpassed that of BL after

all studied growth periods (24, 48 and 72 h). The maximum mean α -amylase activity of BLm, was recorded after 72 h (Fig. 7).

5-Media effect on Bacterial strains

Culture media with or without sugar crop waste were used to spread *Bacillus* strains, and subsequently, amylase activity was measured.

The data in Fig. 7 explored that the best bacterial rendering obtained with sugarcane bagasse and/or sugarbeet pulp supplemented media. The highest level of amylase activity was recorded on M5 medium, which contained both sugarcane bagasse and sugarbeet pulp instead of LB media. The maximum amylase activity was recorded after incubation for 72 h in M4 media (LB broth + sugarcane bagasse), whereas the lowest activity recorded for BLm was obtained after 24 hrs of incubation in LB broth + starch media. These results revealed that the amylase activity of BLm surpassed that of BL in all studied media.

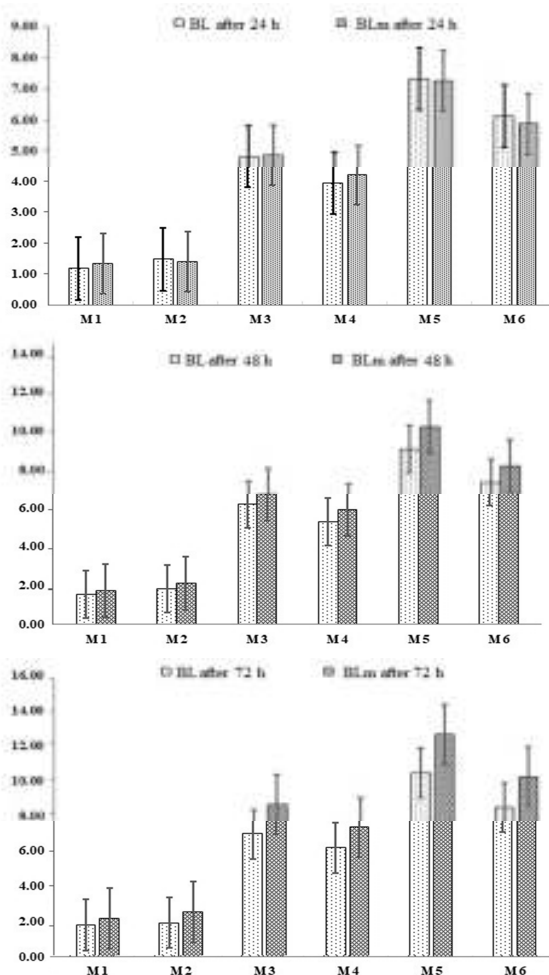


Fig. 7. α -Amylase activity (unit/mL) of *Bacillus licheniformis* (BL) and modified *Bacillus licheniformis* (BLm) over three incubation periods (24, 48 and 72 h) on different media composition

6-Enzyme thermostability

The thermostability of the enzyme against high temperatures (65°C, 80°C and 90°C) was determined at various time durations from 2 h to 24 h (Fig. 8). From this

Figure it could be indicated that the α -amylase activity in modified *B. licheniformis* (BLm) for all temperatures and all times was higher than original *B. licheniformis* (BL).

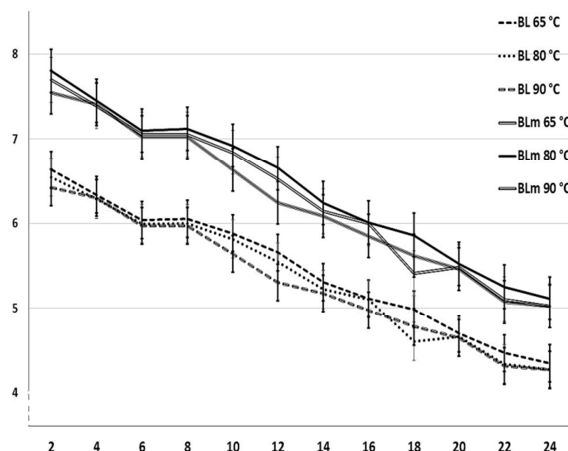


Fig. 8. α -Amylase activity (unit/mL) of original *B. licheniformis* (BL) and modified *B. licheniformis* (BLm) were exposed to different temperature conditions and incubated for 24 h.

Over time, slightly decreases in enzyme activity rates were observed compared with initial time. These decreases ranged from 7.01 % to 8.75% after 8 h; from 22.08 % to 22.95% after 16 h; from 33.49 % to 34.81 % after 24 h in BLm and BL at the different high temperatures. Also, Fig. 9 indicates that the enzyme activity of the modified bacteria was high for 8 consecutive hours at 90 °C. Where the enzyme efficiency was 93.0 % after 8 hours and decreased enzyme efficiency to 66.5 % at the end of 24 hours at 90 °C.

7-Cell Mass amount

Amount of biomass produced by BLm and BL on different media composition was varied (Fig. 9). Out of six tested media and/or reference medium (LB), medium with sugarcane and/or sugarbeet wastes (M3, M4, M5 and M6) had high biomass yield (g/L) (Fig. 9) and M5 recorded the highest one. Interestingly, about 30% of initial starch with LB media (M2) was remaining without being decomposition even after incubation prolonged (72 h).

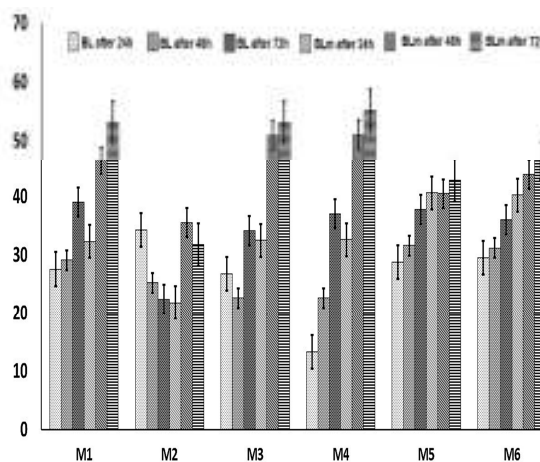


Fig. 9. Biomass production of original and modified *B. licheniformis* in different media over time

DISCUSSION

A gene (BSK1) encoding *Bacillus stearothermophilus* α -amylase (isolated from sugar beet juice of Daqahlia Sugar Factory in Egypt) was cloned into *E. coli* and successfully expressed in *Bacillus licheniformis*. The BSK1 gene expressed a polypeptide with a molecular weight (mol. Wt) of 61.963 kD. The size of obtained polypeptide is similar to some microbial α -amylases (Vihinen and Mäntsälä, 1989) and typical microbial α -amylase such as the amylase from *Bacillus stearothermophilus* isolated by Nakajima et al. (1985) and *Geobacillus stearothermophilus* by Suominen et al. (1987). It is one of large amylases, such as the amylase of *Bacillus polymyxa* (Uozumi et al., 1989), the G6-amylase of *Bacillus sp.* strain H-167 (Shirokizawa et al., 1990) and the G4-amylase of a *Micrococcus sp.* (Kimura and Horikoshi, 1990).

The alignment of the amino acid sequence of *Bacillus stearothermophilus* α -amylase with other amylases showed some highly conserved regions in all enzymes.

Bacillus licheniformis often staked intracellularly insoluble bulks due to the heterologous proteins high-level production, these bulks definite as inclusion bodies. Some scientists did significant efforts to make the express of recombinant proteins in form that could be more soluble and more active (Lunn et al., 1986 ; Goloubinoff et al., 1989 ; Takagi et al., 1990). Our investigation suggests that the BSK1 overexpression in *Bacillus licheniformis* resulted due to inclusion bodies formation. Although soluble proteins have advantages in the production (Shatzman, 1990), inclusion bodies have advantage in their amenable readily to purification and they are stabilize proteins against degradation by host proteases (Cheng et al., 1981). We have purified the recombinant BSK1 with a yield of 1.1302 mg/ml of media.

The recombinant α -amylase was highly active at 65°C as optimum temperature (Fig. 8). The α -amylase of other thermophilic was reported to be less thermostable with optimum activity at 50°C, 55°C, and 60°C, respectively (Aguilar et al., 2000). On the other hand, recombinant α -amylase of *G. stearothermophilus* was found to be thermophilic exhibiting optimum activity at 65°C (Yang et al., 2010). Also the α -amylase retained its maximum activity (over 80%) at 80°C (Fig. 8). Kolcuoğlu et al. (2010) reported that about 80% enzyme activity resulted by incubation at 80°C/72 h., also Ali et al. (1999) demonstrated that the activity is completely stable for at least 90 minutes at 80°C. from Fig. 8 indicating that the thermostability of α -Amylase was not clearly different between BLm and BL, although the activity of the enzyme in BLm was higher than BL. These results provided evidence that gene transfer to BL effectively increased its amylase activity but did not affect its thermostability. The enzyme produced in this study have high thermostability even 90 °C, high optimum and thermostability range allows it to be utilized in industrial applications, because their high yield, as well as their time and cost saving.

Out of six tested media and/or reference medium (LB), medium with sugarcane and/or sugarbeet wastes (M3, M4, M5 and M6) had high biomass yield (g/L) (Fig. 8) and M5 recorded the highest one. Interestingly, about

30% of initial starch with LB media (M2) was remaining without being decomposed even after incubation prolonged (72 h). Our observations found that addition of starch as carbon source had no significant positive effect on biomass yield. On the other hand, sugarcane and sugar beet wastes produced biomass at par with LB medium.

According to our results, sugar crops wastes should be added to the growth medium to obtain highly effects on the *Bacillus* growth, through significantly stimulation the biomass production.

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التحول الوراثي لبكتيريا *Bacillus licheniformis* بالجين المسنول عن إنتاج إنزيم ألفا أميليز في بيئات تحتوي على مخلفات المحاصيل السكرية

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ترجع أهمية إنزيمات الأميليز لقدرتها على تحفيز تحلل النشا والسكريات العديدة إلى جلوكوز ومالتوز ومالتودكستريز والتي تستخدم في العمليات الصناعية. يعود ثبات واستقرار هذه الإنزيمات تحت ظروف عملية مختلفة إلى قدرتها على تحمل الحرارة. وإنتاج الأميليز يحدث في كل الكائنات الحية (البكتيريا والفطريات والنبات والحيوان). في هذه الدراسة تم عزل المنطقة الجينية المسنولة عن إفراز إنزيم ألفا أميليز من بكتيريا *Bacillus stearothermophilus* والتي تتواجد في عصير بنجر السكر وتحمل درجات حرارة عالية أثناء عملية تصنيع السكر حيث تم نقل الجين المتحصل عليه إلى خلايا بكتيريا *Bacillus licheniformis*. وقد أظهرت النتائج أن تتابع هذا الجين كان حوالي 1827 زوج من القواعد وقد تم تسجيله في بنك الجينات تحت رقم M57457.1. كما أظهرت النتائج تشابه تتابع الأحماض الأمينية للإنزيم المعدل بنسبة 98% مع تتابع الإنزيم الموجود في قاعدة بيانات بنوك الجينات. من ناحية أخرى فإن إنزيم ألفا 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 حيث أعطت أعلى معدل نشاط إنزيمي وأكبر كمية من كتلة الخلايا. أيضاً ومن حيث الثبات الحراري للإنزيم فلم يكن مختلفاً بشكل واضح بين البكتيريا المعدلة والبكتيريا الأصلية. وهذه النتائج المتحصل عليها أعطت دليلاً على أن الجين المنقول إلى *Bacillus licheniformis* قد أدى دوراً فاعلاً في زيادة النشاط الإنزيمي للألفا أميليز ولم يكن مؤثراً على ثباته الحراري. الإنزيم المنتج في هذه الدراسة يتميز بثبات حراري حتى 90 م°، وارتفاع مدى الثبات الحراري يسمح باستعمال هذه البكتيريا في التطبيقات الصناعية، وذلك بسبب إنتاجيتها العالية، فضلاً عن توفير الوقت والتكلفة.