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GENETIC IMPROVEMENT OF ALKALINE PROTEASE PRODUCTION VIA *BACILLUS* PROTOPLAST FUSION

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ABSTRACT

This study aimed to improve the production of alkaline protease by *Bacillus* strains through protoplast fusion technique. Different *Bacillus* strains were screened for alkaline protease production. The best producers were *B. alvei* and *B. licheniformis* (local isolates) were fused together.

Twelve fusants were selected on the basis of antibiotic resistance pattern. All fusants were characterized on the basis of enzyme activity, resistance to antibiotics and different chemical and physical factors. Plasmid profiles studies of these fusants and their parents showed the transfer of some plasmids from each parent to the fusants. All fusants showed higher efficiencies in enzyme productivity than their parents. Up to five times alkaline protease activity were found in fusants comparing with their parental strains production.

The distinguished obtained fusants are available to be used commercially in many economic important industries.

Keywords: *Bacillus* spp., Genetic improvement, Protoplast fusion, Alkaline protease.

INTRODUCTION

Proteases are one of the most important industrial enzymes where it represents nearly 60 % of the total worldwide enzymes sales. Of these, alkaline proteases are employed primary as cleansing additives (Kunamneni *et al.*, 2003).

Bacteria of the genus *Bacillus* are active producers of Extracellular alkaline proteases. Alkaline proteases are one of the most widely studied enzymes group because of their usage in many industrial applications (Jasvir *et al.*, 2004).

Protoplast fusion is a versatile technique for inducing genetic recombination in a variety of prokaryotic and eukaryotic microorganisms, such as *Bacillus*. Protoplasts are prepared by treating bacteria with a lytic enzyme such as lysozyme that removes the cell wall. As a result of this treatment, the cell content would be enclosed only by the cell membrane. The protoplasts have to be preserved in a hypertonic medium for their osmotic stability and survival. Then, in the presence of fusogenic agent such as polyethylene glycol (PEG), protoplasts are induced to fuse and form transient hybrids. During this hybrid state, the genomes may re–assort and genetic recombination can occur (Shamsi *et al.*, 2002). So far, an increasing number of recombinant strains have been formed.

This study aimed to improve alkaline protease production of two *Bacillus* producer strains via protoplast fusion technique. The promising strains in this study could be used directly in many economic important industries.

MATERIALS AND METHODS

Materials:

Bacterial strains:

Four *Bacillus* strains, i.e., *B. alvei* , *B. licheniformis* ,*B. pumilus* and *B. cereus* were kindly provided from Agricultural Microbiology Dept., NRC, Dokki, Cairo, Egypt and used through this investigation.

Media:

Lauria – Bertani (LB) broth medium (Davis *et al.*, 1980) was used to propagate *Bacillus* strains at 37^{0}C with vigorous aeration.

Nutrient Yeast Salt Medium (NYSM) broth was used in alkaline protease production (Yousten and Davidson, 1982).

LB plus 1% skim milk agar (Kunamneni *et. al.*, 2003) was used to detect the production of alkaline protease.

Methods:

Strain characterization was studied according to Gordon *et al.*, 1973.

Antibiotic resistance test was done according to Jandova and Tichy (1987).

Alkaline protease detection on agar plates was carried out according to Kunamneni *et. al.* (2003), where a clear zone of skim milk hydrolysis gave an indication of protease production after incubation at 37^{0}C for 40 hours under alkaline conditions.

Alkaline protease activity was assayed according to the method of Dumusois and Priest (1993) using Azocasein (0.4 %) as a substrate. One enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm /30 min. under standard reaction conditions.

Soluble protein was determined by the method of Ohnistti and Barr (1978) using bovine serum albumin as a standard.

Protoplast fusion was carried out according to Shamsi *et al.* (2002).

Plasmid isolation was done using the miniscreen method of Rodriguez and Tait (1983).

Plasmid analysis was performed by agarose-gel electrophoresis according to Sambrook *et al.* (1989).

RESULTS AND DISCUSSION

Characterization and determination of enzyme activity of *Bacillus* strains:

The four *Bacillus* strains were tested for their sensitivity against the two antibiotics ampicillin or tetracycline. Their antibiotic resistance patterns are present in Table 1. These strains were screened for their alkaline protease production at pH 9 following the method of Dumusois and Priest (1993) using 0.4% Azocasein as a substrate.

The alkaline protease production of the *Bacillus* strains are present in Table 1. Table 1 also presents the response of the tested strains to different

chemical and physical factors, i.e., citrate utilization, growth at 50°C, growth in 7% NaCl and nitrate reduction.

Table (1): Alkaline protease activity of *Bacillus* strains and their characteristics.

Strains	1	2	3	4	5	Antibiotic resistance	
						Ap	Tc
B. alvei	100.5	--	--	--	--	--	+
B. licheniformis	186.4	+	+	+	+	+	--
B. pumilus	60.3	+	--	--	--	+	+
B. cereus	32.6	+	--	--	+	+	+

(1) Alkaline protease activity U/ml. (2) Citrate utilization. (3)Growth at 50°C. (4)Growth in 7 % NaCl. (5) Nitrate reduction.

One enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm /30 min. under standard reaction conditions.

Protoplast fusion:

Protoplast fusion technique is used to hybrid two or more species which could not be obtained by classical mating. Intergeneric or interspecific fusion provides a method for the introduction and transfer of desirable genes, e.g., protease genes. *Bacillus alvei* and *B. licheniformis* were chosen for genetic improvement attempts via protoplast fusion due to their higher alkaline protease production.

The characters of the *Bacillus* parent strains were shown in Table (1). Protoplasts were induced from both selected strains. They were mixed with PEG as the fusogenic agent. For the selection of fusants, samples of 100 µl of the protoplast suspension were added to LB medium supplemented with Tc and Ap. Cells that resisted both antibiotics, AP and Tc, were selected as fusants.

Table (2): Characterization of *B.alvei, B. licheniformis* and their fusants.

Strains	1	2	3	4	5	Antibiotic resistance	
						Ap	Tc
B. alvei	100.5	-	-	-	-	-	+
B. licheniformis	186.4	+	+	+	+	+	-
BAL1	316.8	+	+	+	+	+	+
BAL2	518.0	+	+	+	+	+	+
BAL 3	486.0	+	+	+	+	+	+
BAL4	504.0	+	+	+	+	+	+
BAL5	488.0	+	+	+	+	+	+
BAL6	444.0	+	+	+	+	+	+
BAL7	450.0	+	+	+	+	+	+
BAL 8	469.0	+	+	+	+	+	+
BAL 9	360.0	+	+	+	+	+	+
BAL10	443.0	+	+	+	+	+	+
BAL11	460.0	+	+	+	+	+	+
BAL12	510.0	+	+	+	+	+	+

(1) Alkaline protease activity U/ml. (2) Citrate utilization. (3)Growth at 50°C. (4)Growth in 7 % NaCl. (5) Nitrate reduction.

One enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm /30 min. under standard reaction conditions.

Twelve genetically stable fusants were obtained they were capable to grow on 7% NaCl while the parent *B.alvei* can not grow at the same level of salinity (Table 2).

Results represented in Tables (2 and 3) proved that all fusants were more efficient in alkaline protease activity than their parents. The best enzyme producers were BLA 2, BLA 12 and BLA 4 where the enzyme activity reached 518, 510 and 504 U/ml, respectively.

On the other hand, the enzyme activity was increased 5.01, 5.07 and 5.15 times in fusants No. BLA 4, 12 and 2, respectively, than the parent *B. alvei* while they were increased 2.7, 2.74 and 2.78 times than the other parent *B. licheniformis* . Results also showed that all obtained fusants had acquired resistance for both antibiotics from the two parents. The fusants had acquired the same characters pattern of the parental strain *B. licheniformis* , i.e., citrate utilization, growth at 50°C, growth in 7% NaCl and nitrate reduction (Table 2). This finding indicated that the fusants' genetic backgrounds are mainly from *B. licheniformis* parental strain.

Table (3): The alkaline protease activity of fusants strains at pH 9:

Fusants No.	Enzyme activity U/ml	Protein mg/ml	Specific activity U/mg protein
BAL1	316.8	0.68	617.3
BAL2	518.0	0.63	822.2
BAL3	486.0	0.62	783.8
BAL4	504.0	0.56	900.0
BAL5	488.0	0.65	750.7
BAL6	444.0	0.67	662.6
BAL7	450.0	0.67	671.6
BAL8	469.0	0.62	756.4
BAL9	360.0	0.71	507.0
BAL10	443.0	0.63	703.2
BAL11	470.0	0.56	821.4
BAL12	510.0	0.57	894.7

One enzyme unit was defined as the amount of enzyme that yields an increase of 0.1 OD at 420 nm /30 min. under standard reaction conditions. Specific activity is expressed as U / mg protein.

Plasmid profiles of *B. alvei* and *B. licheniformis* and their fusants:

Plasmids of parent strains and their fusants were isolated and visualized via agarose gel – electrophoresis. Results are present and illustrated in (Fig.1). All plasmids detected were of high molecular weight comparing with λ DNA marker digested with *Hind* III (M). Fig. (1) Showed that *B. alvei* (lane 1) had five plasmids while the other parent, *B. licheniformis* (lane 2) had only three plasmids.

Results also showed that, the number of plasmids in all fusants was ranged from three to five. Four fusants; lanes 6,9,12 and 14; had three

plasmids while six fusants; lanes 3, 7, 8, 10,11and 13; had four plasmids. On the other hand, two fusants; lanes 4 and 5; showed the highest plasmid number, i.e., five plasmids.

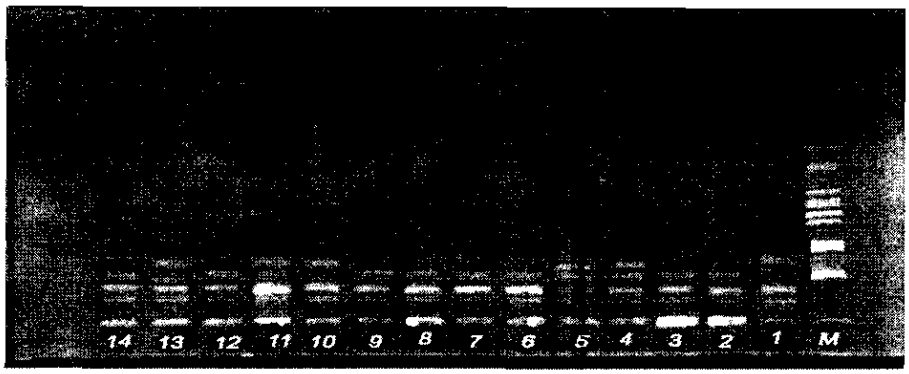

Fig. (1): Plasmid profiles of the two parents, *B. alvei* (lane 1) and *B. licheniformis* (lane 2), and their twelve fusants (lanes 3 to 14).

Plasmid patterns studies indicated the successful transfer of genetic materials from both parental strains to their fusants, i.e., each fusant has acquired certain plasmid(s) from each parent. Fusants No. BAL1, BAL12 and BAL4; lanes 4 14 and 6; had acquired plasmid band identical to that present in the parent *B. licheniformis* . This plasmid harboring gene(s) responsible for the increasing in production level of alkaline protease by increase the copy number óf the protease gene (s) and these results were in agreement with results reported by Jorgensen *et al* . 2000.

On the other hand, results indicated the presence of plasmids containing Ap^r gene(s) which were coded by chromosomal gene (Solaiman *et al.*, 2003) in all fusants acquired from the parent *B. licheniformis* . These results also showed the successful replication and maintenance of this plasmid in all fusants obtained when they grown in the selective medium supplemented with ampicillin. Results in the rest obtained fusants did not show the presence of some kind of plasmids from both parents. It may be suggested that the increasing which detected in enzyme activity in these fusants can be resulted from elimination of repressor gene(s) located on these plasmids which repressed chromosomal genes that responsible for productivity (Solaiman *et al.*, 2003). This finding indicated that the fusants' genetic backgrounds are mainly from *B. licheniformis* parental strain.

In conclusion, interspecific protoplast fusion technique is a powerful method for improving alkaline protease production in *Bacillus*. The obtained fusants through this study are more efficient in alkaline protease activity than their parents and it is recommended to use these new improved strains commercially in many economic important industries.

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التحسين الوراثى لانتاج الإنزيم القلوي المحلل للبروتين بواسطة الدمج البروتوبلاستي للباسيلاس

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تهدف هذه الدراسة إلى التحسين الوراثى لإنتاجية بعض سلالات من جنس الباسيلاس للإنزيم القلوي المحلل للبروتين من خلال الدمج البروتوبلاستي.

تم عمل دمج بروتوبلاستى بين سلالتين محليتين من جنس الباسيلاس ذات إنتاجيه عالية للإنزيم. وقد تم الحصول على ١٢ مندمجة باستخدام صفة المقاومة للمضادات الحيوية كمعلمات وراثية. تم توصيف المندمجات على أساس إنتاجيتها للإنزيم القلوي المحلل للبروتين ومقاومتها لبعض المضادات الحيوية بالإضافة إلى بعض التفاعلات البيولوجية وقد أظهرت هذه المندمجات تفوقا فى إنتاج الإنزيم القلوي المحلل للبروتين وصل فى بعضها إلى خمسة أضعاف ما ينتجه الآباء.

وبدراسة نماذج البلازميدات بطريقة التفريد الكهربي لكل المندمجات ومقارنتها بآبائها أتضح انتقال بعض البلازميدات من كلا الآبوين إلى المندمجات. وتفسير زيادة كفاءة المندمجات فى إنتاج الإنزيم قد يرجع إلى تزايد أعداد الجينات المسئولة عن هذا الإنتاج سواء ما تحملة البلازميدات من جينات أو تلك الموجودة على الكر وموسوم.

ويوصى باستخدام هذه المندمجات المتميزة ذات الانتاجيه العالية والتي حصلنا عليها فى هذه الدراسة تجاريا لما لها من أهمية تطبيقية فى بعض الصناعات الاقتصادية الهامة.