

## Enhanced Production of Extra Cellular Alkaline Protease in *Bacillus Circulance* Through Plasmid Transfer

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**Abstract:** Five local strains of *Bacillus* spp. were screened for alkaline protease production. They were *Bacillus circulance*, *Bacillus cereus*, *Bacillus alvei*, *Bacillus sphaericus* and *Bacillus pumilus*. *Bacillus alvei* revealed the highest alkaline protease production, while *Bacillus circulance* had the lowest productivity among all strains. All strains were characterized on the basis of resistance to either ampicillin (Ap) or tetracycline (Tc) antibiotics as a genetic marker. *Bacillus* strains were resistant to both antibiotics, (Ap) and (Tc), except *Bacillus circulance* which was sensitive to both. Due to their antibiotic resistant patterns, all strains were used as sources of plasmids (donor strains) to transform *Bacillus circulance* to study their effect in alkaline protease productivity. Eight *Bacillus circulance* transformants were isolated and selected according to their antibiotic resistance. Variation in alkaline protease production levels was detected in all transformants, i.e., 14.7 to 330 U/ml. Up to seven and half times more alkaline protease activity were found in transformants comparing with the recipient strain production. The three highest transformants in enzyme production were BC.NRC4, BC.NRC3 and BC.NRC5, i.e., 330, 305 and 227.5 U/ml, respectively. Plasmid profile studies of recipient strain, *Bacillus alvei*, *Bacillus sphaericus* (donor strains) and the three highest transformants were done. Results confirmed that transfer of plasmids to these transformants. The promising genetically improved strains produced in this study could be used commercially and the system described here could be used also in higher alkaline protease production of other *Bacillus* strains.

**Key words:** *Bacillus*, transformation, alkaline protease.

### INTRODUCTION

The detergent industry is the largest user of industrial enzymes. Alkaline proteases are one of the most widely studied groups of enzymes because of their use in many industrial applications<sup>[7]</sup>. The enzymes used in the detergent industry today are mainly proteases and all from *Bacillus* origin<sup>[15]</sup>. Many *Bacillus* proteases have been characterized and their encoding genes have been cloned and sequenced<sup>[8,19,12]</sup>. The *aprN* gene consisted of 1137 bp encoding protein of 376 amino acids organized into a single peptide (29 amino acids), a pro-protein (76 amino acids), and mature enzyme (274 amino acids)<sup>[5]</sup>. *Bacillus* species carrying *subC* gene encoding serine alkaline protease (SAP) enzyme were developed in order to increase the yield and selectivity in the bioprocess for (SAP) production, for this aim, *subC* gene was cloned into pHV 1431 Escherichia coli -*Bacillus* shuttle vector, and transferred into nine host *Bacillus* species.

Among the *Bacillus* species, the highest volumetric (SAP) activity was obtained with some transformed species while the lowest was obtained with some other species<sup>[2]</sup>. Genetic transformation is the process through

which exogenous DNA is introduced into a receiving cell to permanently alter its heredity. To overproduce the extra cellular *Bacillus* protease, two strategies for increasing the corresponding gene copy number can be considered: (1) using plasmid – containing strains and (2) using strains containing additional genes integrated in their chromosome. In this study, we aimed to introduce different types of plasmids, isolated from different *Bacillus* species, into *B. circulance* to obtain high efficient enzyme activity strains.

This study aimed to construct over producer alkaline protease *Bacillus* strains through plasmid transfer system which could be used in many economic applications.

### MATERIALS AND METHODS

#### Materials:

**Bacterial Strains:** Five *Bacillus* strains; *Bacillus circulance*, *Bacillus cereus*, *Bacillus alvei*, *Bacillus sphaericus* and *Bacillus pumilus* which produce alkaline protease under alkaline conditions<sup>[17]</sup>, were obtained from Microbial Genetics Dept., NRC, Cairo, Egypt and used in this study.

#### Media:

- Luria- Bertani broth (LB) and (LB agar) media<sup>[20]</sup> were used to propagate *Bacillus* strains at 37°C with vigorous aeration. LB agar medium was supplemented with appropriate concentrations of the corresponding antibiotics when necessary for antibiotic resistance test and for the selection of transformants.
- LB agar plus 1% skim milk<sup>[11]</sup>, was used to detect the production of alkaline protease, i.e., a clear zone of skim milk hydrolysis gave an indication of protease production after incubation at 37°C for 40 hours under alkaline conditions.
- Nutrient Yeast Salt (NYSM) broth medium was used in alkaline protease production<sup>[20]</sup>.

#### Methods:

- Alkaline protease detection was carried out according to<sup>[11]</sup>.
  - Alkaline protease activity using (0.4%) azocasein as a substrate, was determined according to the method of<sup>[4]</sup> whereas one enzyme unit was defined as the amount of enzyme that yields an increase of 0.1OD at 420 nm/30 min. under alkaline conditions.
  - Antibiotic resistance test was done according to Jandova and Tichy<sup>[6]</sup>.
  - Plasmid isolation was done using the miniscreen method of Rodriguez and Tait<sup>[14]</sup>.
  - Plasmid analysis was performed by agarose-gel electrophoresis according to Sambrook *et al*<sup>[16]</sup>.
  - Transformation experiments were done as described by Ausubel<sup>[1]</sup>.
- Experiments were carried out in triplicate.

## RESULTS AND DISCUSSION

**Genetic Marker and Enzyme Activity of *Bacillus* Strains:** The presence of certain genetic marker is an important tool for tracing genetic characters through genetic experiments. Therefore, the previous five *Bacillus* strains were tested for their sensitivity against two antibiotics, ampicillin (Ap) and tetracycline (Tc). Their antibiotic resistance patterns are presented in (Table 1). These strains were also screened for their alkaline protease production at pH 9 in triplicate and results were represented in (Table 1).

Results in Table (1) proved that all tested strains were resistant to Tc and Ap except *Bacillus circulance* which was sensitive to both antibiotics. Results also showed a broad range of alkaline protease productivity among the tested strains. The highest productive strain was *B. alvei* which gave 100.5 U/ml.

**Transformation of *Bacillus circulance*:** Some *Bacillus* spp. are the preferred work – horse due to their strains

capability to become competent for the uptake of plasmid DNA as well as chromosomal DNA with high frequencies. Plasmids which contain genes encoding novel enzymes activities are maintained by the use of antibiotic resistance selection and which frequently have a high copy number to ensure maximal production of the enzyme. Transformation of competent cells of *B. circulans* was performed as described by Ausubel *et al*<sup>[1]</sup>. *B. circulans* was chosen as recipient strain due to its sensitivity against the two tested antibiotics and to examine the effects of exogenous plasmids in alkaline protease production. The rest *Bacillus* strains were used as donors of plasmids DNAs. *B. circulans* competent cells were prepared as outlined by Ausubel *et al*<sup>[1]</sup>. Plasmids of *Bacillus* donor strains were isolated by using the miniscreen method of Rodriguez and Tait<sup>[14]</sup>. Fifteen µl of plasmid DNA (10ng) were added in a 15 ml test tube and kept on ice. The competent cells were rapidly thawed and 100µl was quickly dispensed into the test tube. It was kept on ice for 30 min. and the mixture was then exposure to a heat shock at 42°C for 2 min. Adding 1 ml of LB broth and incubated at 37°C in a shaker incubator (200rpm) for 0.5 and 1 h.

To isolate transformants, 100µl of appropriate dilution were spread on LB agar selective medium supplemented with appropriate concentrations of selection marker (Ap and Tc) and 1% skim milk. Transformants were selected on the basis of antibiotic resistance pattern. Cells that resist Ap and Tc antibiotics were selected as transformants. Controls run without plasmid DNA produced no colonies on selective medium.

Eight *B. circulans* transformants were successfully isolated, their names and enzyme activities are presented in Table (2).

Alkaline protease activity of *Bacillus* transformants showed different levels of enzyme activities ranged from 14.7 to 330 U/ml. The highest producer transformants were BC.NRC4, BC.NRC3 and BC.NRC5, i.e., 330, 305 and 22.5 U/ml, i.e., 7.5, 6.9 and 5.1 more times that of the recipient strain, respectively. BC.NRC1 and BC.NRC2 were the lowest in productivity and this might be due to that transformation occurs without expression of alkaline protease gene(s) in its new host. On the other hand, the noticed decrease in alkaline protease productivity detected for BC.NRC1 and BC.NRC2 transformants may be due to any of the following possibilities; natural regulatory relations between indigenous existence plasmids and chromosomal gene or presence of certain genes located on transferred plasmids which have a negative influence on recipient chromosomal genes that responsible for productivity, i.e., repression<sup>[17]</sup>. Uehara *et al*<sup>[18]</sup> reported the presence of regulatory genes responsible for the production of alkaline protease. Knceroa *et al*<sup>[10]</sup> reported

**Table 1:** The genetic marker and alkaline protease activity of *Bacillus* strains.

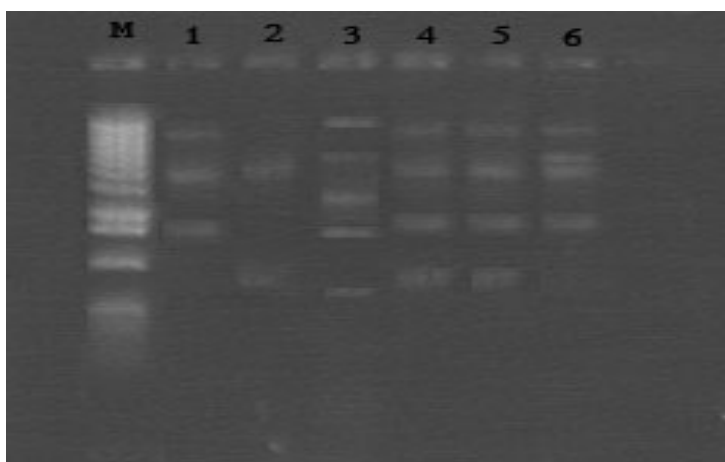
Strains	Genetic marker	Enzyme activity U / ml
<i>B. circulans</i> *	Tc <sup>s</sup> , Ap <sup>s</sup>	44.2
<i>B. cereus</i>	Tc <sup>r</sup> , Ap <sup>r</sup>	60.3
<i>B. alvei</i>	Tc <sup>r</sup> , Ap <sup>r</sup>	100.5
<i>B. sphaericus</i>	Tc <sup>r</sup> , Ap <sup>r</sup>	50.4
<i>B. pumilus</i>	Tc <sup>r</sup> , Ap <sup>r</sup>	60.3

(Tc<sup>r</sup>)Tetracycline resistance - (Tc<sup>s</sup>) Tetracycline sensitive  
(Ap<sup>r</sup>) Ampicillin resistance - (Ap<sup>s</sup>) Ampicillin sensitive

**Table 2:** The alkaline protease activity of *Bacillus circulans* transformants.

Recipient strain	EA	Donors strains	EA	Transformants	EA
<i>B. circulans</i>	44.2	<i>B. cereus</i>	60.3	BC.NRC1	14.7
				BC.NRC2	22.6
		<i>B.sphaericus</i>	50.4	BC.NRC3	305
				BC.NRC4	330
		<i>B. alvei</i>	100.5	BC.NRC5	227.5
				BC.NRC6	184.5
		<i>B. pumilus</i>	60.3	BC.NRC7	180.3
				BC.NRC8	190

EA: Enzyme activity U/ml



**Fig. 1:** Agarose gel electrophoresis of plasmids isolated from recipient, donors and three transformants strains.

- Lane (M)  $\lambda$  DNA digested with Hind III
- Lane (1) *B. circulans* (recipient)
- Lane (2) *B.sphaericus* (donor)
- Lane (3) *B. alvei* (donor)
- Lane (4) BC.NRC4
- Lane (5) BC.NRC3
- Lane (6) BC.NRC5

that alkaline protease production is a multigenic controlled system which is in accordance with the present findings.

**Plasmid Profiles of Recipient, Two Donors and the Highest Three Transformants: *B. circulans* (recipient**

strain), *B.sphaericus*, *B. alvei* (donor strains) and the highest three transformants, i.e., BC.NRC4, BC.NRC3 and BC.NRC5 were chosen for plasmid profile determination. Results showed that plasmid DNAs isolated from transformants were indistinguishable by molecular weight from those used for transformation.

The plasmids were stable in *B. circulans* transformants, expressing their resistance marker for Ap after growth of transformants because Ap resistance gene(s) were coded by chromosomal gene(s) as reported by Peter *et al*<sup>[13]</sup> which is in accordance with the present findings. Plasmids of recipient, two donors and the highest three transformants were isolated and visualized via agarose gel electrophoresis. Results are represented and illustrated in Fig. (1). Plasmid profile studies showed that *B. circulans* recipient strain (Lane 1) had three different molecular weight plasmids, *B. sphaericus* (Lane 2) donor strain had two plasmids, while *B. alvei* (Lane 3) had five plasmids. Results also showed that, the number of plasmids in all transformants was four plasmids.

Plasmid patterns studies indicated the successful transfer of plasmids from donor strains to their transformants, i.e., each transformant has acquired certain plasmid(s) from each donor strain. The BC.NRC3 and BC.NRC4 transformants (lane 5 and 4) contain the three original *B. circulans* plasmids (B.C.PI, PII and PIII) and also (B.S.PII) plasmid from *B. sphaericus*. Results also showed that BC.NRC5 transformant (Lane 6) was successful uptake plasmid (B.A.PII) from *B. alvei* donor strain (lane 3). These plasmids harboring gene(s) responsible for increasing the production level of alkaline protease and this results were in agreement with results reported by<sup>[9]</sup>. On the other hand, results indicated that antibiotic resistance Ap<sup>r</sup> and Tc<sup>r</sup> gene(s) were found in all transformants acquired from donor strains.

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