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COMPARISON SURVEY OF RECEIVING THE PLASMID 253PIL BY BACILLUS CEREUSATCC1098 AND BACILLUS CEREUS ATCC14579 BY USING OF ELECTROPORATION AND THERMAL SHOCK METHOD

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ABSTRACT

According to the studies conducted, Bacillus cereus is a gram-positive aerobic bacterium which is able to produce spores. B. cereus is involved in formation of microflora. Currently, some strains of B. cereus are used as probiotics in animal and poultry feed. Genetic investigation is considered as an important objective of biotechnological and microbiological studies. This study compares the received plasmid 253pIL by B. cereus ATCC14579 and B. cereus ATCC1098. There are several methods for transformation; this study used chemical method (thermal shock) and electroporation. Due to low efficiency of these two methods in B. cereus strains, the protoplast of two B. cereus strains ATCC14579 and ATCC10987 were used in this study. Finally, these two strains were compared for two different transformation methods. The results showed that both strains ATCC1098 and ATCC14579 received more plasmid via electroporation.

Keywords: Bacillus cereus, Electroporation, Thermal shock, Transformation, Heat shock, Plasmid.

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Contribution/ Originality

This study contributes in the existing literature about the transformation procedure. This study uses new estimation methodology for comparing two method of transformation in Bacteria. This study originates new formula for evaluation efficacy of transformation.

1. INTRODUCTION

The phenomenon of transformation was first reported In 1928 for Streptococcus pneumonia [1]. Through transformation, foreign DNA will be uptaken by a contacting competent cell [2]. DNA uptake competence is a complex phenomenon; not all cells are competent. In Bacillus subtilis, for example, only 10-20% of the cells can uptake foreign DNA [3]. Bacillus cereus is a Gram-positive bacterium existing in soil and air. B. cereus is an opportunistic pathogen which can cause food poisoning [4]. B. cereus is highly associated with Bacillus thuringiensis. Bacillus mycoides and Bacillus anthracis are causal factors of anthrax [5]. In addition to produce toxins which cause gastroenteritis, B. cereus produces several other virulence factors which are responsible for non gastroenteritic infections. Virulence factors of B. cereus include hemolysin, phospholipase, collagenase, protease and β-lactamase [6]. Genetic engineering and different gene transformation techniques allow the transformation of genes from any species to another. Gene transformation techniques include indirect transmission (Agrobacterium-mediated

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transformation) and direct transformation (particle bombardment, electroporation, micro and macro injection, liposome-mediated transformation, polycation-mediated transformation). In recent years, efforts have been made to increase success rate of B. subtilis or use a combination of osmoprotective factors and high electrical fields. Similar studies have been conducted for … bacteria using osmoprotectives along with glycine treatment and high electric field [2]. These strategies have been tested for members of the B. cereus group. The highest efficiency of electroporation was 103 cfu.μg^{-1} for B. cereus, which is inadequate for many applications such as mutagenesis [7]. Functions of flagellated B. cereus are still not fully understood in pathogenesis; however, flagella play a role in formation of B. cereus biofilms [8]. Some genome sequences of B. cereus strains have been available since the first genome sequencing of the B. cereus strain atcc14579. The term plasmid was first used by the American biologist [7]. Plasmid is a small DNA molecule which exist in the cell separately from the chromosome. Replication of plasmids occurs in cytoplasm independently. Plasmids are usually circular double-stranded DNA molecules, though there are types of linear plasmid. Each plasmid has an origin of replication (ori) from which plasmid replication starts. Size of plasmids varies from 1 to 1000 bp. A cell may contain one to thousands plasmids. In this study, protoplasts of two B. cereus strains ATCC14579 and ATCC10987 are transformed using plasmid 253pIL by electroporation and thermal shock. This study determines whether the strain ATCC1098 receives more plasmid than the strain ATCC14579 by electroporation, or the strain ATCC1098 receives more plasmid than the strain ATCC14579 by thermal shock.

2. MATERIALS AND METHODS

Materials and devices used in this study include electroporation device (BIO RAD Gene pulserxcell), plasmid pIL253 (Proteomix research center, Life Technologies Inc.), lysozyme (Sigma-Aldrich Company), biophotometer (BioPhotometer plus, Eppendorf), mutanolysin (Sigma-Aldrich Company), the medium DM3 (pan-biotech), the medium SMM (Sigma-Aldrich Company) and pseudomonas agar base (PAB, pan-biotech).

3. METHODS

B. cereus strains ATCC1098 (extracted from cheese) and ATCC14579 (extracted from milk) were cultured in liquid PAB at 37°C to OD600 = 1.7-2. Then, the protoplasts were produced by the method used by Chang and Cohen. In this method, the cultured cells were collected by centrifuging the cell plate. Then, the cell plate was dissolved in a SMPP medium consisting of 2x SMM containing 1 M sucrose, 0.04 M malic acid and 0.04 magnesium chloride (pH = 6. 5). The solution was placed on a shaker at 37°C for 30 minutes in the presence of lysozyme (2.5, 5 and 10 mg/ml) and mutanolysin (75 U/ml) to obtain protoplast. The cells were collected by refrigerated centrifuge (4°C, 5200xg rpm for 5 minutes).

4. MEASUREMENT AND ANALYSIS

4.1. Transformation by Thermal Shock and Electroporation

Once the cells were cultures and the fresh plate of newly grown cells was obtained, 0.1 M calcium chloride solution was used to rinse the bacteria. Each rinsing cycle required 30 minutes of incubation on ice. The competent cell was produced after two rinsing cycles. Then, the plasmid was placed adjacent to competent cells at 42°C (thermal shock) and immediately placed on ice. Next, 1ml SMPP was added to the plasmid and incubated at 37°C for 12 hours (100rpm). Finally, the transformed bacteria was cultured in the medium DM3 containing the proper antibiotic for 48 hours at 37°C. For electroporation, the produced protoplast cells were collected after the centrifuge and rinsed twice with the washing buffer, electro-transform. For this purpose, 108 cells were used. Then, 120 ml protoplast was suspended in 5.2 macro liters (1 mg) plasmid and placed on ice for 5 minutes. Electroporation was done at 7.0 kW, 400 Ω and 25μF by pulsing. Next, 1ml SMPP was added to the plasmid and incubated at 37°C for 12 hours (100rpm). Finally, the transformed bacteria was cultured in the medium DM3 containing the proper antibiotic for 48 hours at 37°C. Transformation efficiency (TE) was measured by Tu method in μl. The formula is:

$$TE = \frac{colonies\ on\ plate}{Ng\ of\ DNA\ plated} * 1000 \frac{ng}{\mu g} \quad (1)$$

5. RESULTS

T-test was used to determine significance of the relationship between strains.

Table-1. t-test between two strains studied

T-Test		
Mean	0.291	0.329
Variance	0.065	0.084
Observation	9	9
T-Test*	0.559	

**P= 0.05*

T-test (student t) will be used to assess goodness of fit of a sample to the population if the standard deviation is unknown. T-test results listed in Table 1 show no uniformity between the means of transformant produced in two different strains extracted from milk and cheese. Thus, there is no significant relationship between these two strains.

5.1. Results of Thermal Shock

Table-2. Number of colonies produced by thermal shock

Thermal shock		
1	Count of colonies	
	Mean	76
	Standard deviation	4.5
2	Plasmid specifications	
	0. 004µg/µl plasmid 200µl CaCl2 250 µl LB broth 10 µl of plasmid added to culture 100 µl of solution from the reaction tube spread on agar plate	

Source: Material and Method

As shown in Table 2, the mean number of colonies produced by thermal shock was 76 (±4.5).

5.2. Results of Electroporation

Table-3. Number of colonies produced by electroporation

Electroporation		
1	Count of colonies	
	Mean	352
	Standard deviation	6.11
2	Plasmid Specifications	
	0. 004µg/µl plasmid 200µl CaCl2 250 µl LB broth 10 µl of plasmid added to culture 100 µl of solution from the reaction tube spread on agar plate	

Source: Result data

As shown in Table 3, the mean number of colonies produced by electroporation was 352 (±6.11).

5.3. Transformation Efficiency

Table-4. Evaluation of TE in the presence or absence of plasmid (PILL 253) for untreated cells

TE (Transformation Efficiency)				
$CFE_{ATCC\ 1098}$	$CFE_{ATCC\ 14579}$	Untreated Cells	Plasmid	Receiver
-	-	$7.5 * 10^5$	None	ATCC 14579
ND	$1.5 * 10^6$	$3.5 * 10^6$	PIL 253	
-	-	$2.5 * 10^5$	None	ATCC 1098
$3.1 * 10^5$	ND	$2.3 * 10^6$	PIL 253	

Source: Result data

As shown in Table 4, TE = 3.5×10^6 for the strain ATCC 14579 isolated from milk for the untreated cells and TE = 2.3×10^6 for the strain ATCC1098 isolated from cheese. Table 5 shows TE for the treated bacterial cells.

Table-5. Evaluation of TE in the presence or absence of plasmid (PILL 253) for treated cells

TE (Transformation Efficiency)				
$CFE_{ATCC\ 1098}$	$CFE_{ATCC\ 14579}$	Treated Cells	Plasmid	Receiver
-	-	$5.5 * 10^5$	None	ATCC 14579
ND	$5.5 * 10^6$	$7.5 * 10^6$	PIL 253	
-	-	$6.4 * 10^5$	None	ATCC 1098
$6.1 * 10^5$	ND	$8.6 * 10^6$	PIL 253	

Source: Result data

As shown in Table 5, TE = 8.6×10^6 for the strain ATCC1098 isolated from cheese for the treated cells and TE = 7.5×10^6 for the strain ATCC14579 isolated from milk. This is different from the previous case were the cells were untreated. The bacterial strains were treated by different concentrations of lysozyme, which result in different growth based on the concentrations used.

6. DISCUSSION AND CONCLUSION

In recent years, efforts have been made to increase success rate of B. subtilis or use a combination of osmoprotective factors and high electrical fields. Similar studies have been conducted for … bacteria using osmoprotectives along with glycine treatment and high electric field. These strategies have been tested for members of B. cereus group. The highest efficiency of electroporation was 103 $cfu.\mu g^{-1}$ for B. cereus, which is inadequate for many applications such as mutagenesis [3]. Bacterial transformation was first described by Greif in 1928 when DNA was known as a genetic material. Greif observed that combination of the killed pathogenic Pneumocy cells with strain of non-pathogenic living cells r could permanently transform the rough strain (r) to the malignant cell (s). It was confirmed that transformation was based on high-quality DNA isolated from r-cells; therefore, new genetic characteristics were acquired. Members of at least 15 genera of bacteria are naturally high quality. Current knowledge about natural process of DNA uptake has been studied in the literature. Very few systems have been described for bacterial transformation. The correct conditions must be provided for bacterial transformation; since a limited number of bacteria are naturally high quality, few numbers of bacterial transformation systems are known. Strains of Agrobacterium which lack the plasmid Ti are not able to cause gall in the plant.The first step in transformation of T-DNA to the plant is bacterial uptake on the wounds on the stem and crown of the plant. In fact, it is assumed that the wounds eliminate the physical obstacles of bacterial transformation into the plant, thereby facilitate T-DNA transformation. Currently, it has been proved that bacteria react under the influence of some phenolic compounds, such as Hydroxyacetosyringone and Acetosyringone secreted from injured tissue. These materials, in fact, are part of the metabolic products of phenylpropanoids, producing secondary metabolites such as lignin and flavonoids. Phenolic

compounds stimulate activity of some genes on the plasmid Ti and play an important role in transformation of T-DNA to the plant. This group of genes is called virulence gene or Vir. Virulence genes are located on an area of the plasmi Ti with 35 kbp in length. Products of virulence genes are essential for transformation and replacement of the T-DNA into the plant genome. For induction of transformation, T-DNA and virulence genes are not required to locate on a single Ti plasmid. A bacterium containing plasmid and virulence genes and another bacterium containing T-DNA and the considered gene will be able to transform T-DNA into the plant genome successfully. By reviewing literature and analyzing data, this section discusses transformation of protoplasts of two B. cereus strains ATCC14579 and ATCC10987 using the plasmid 253pIL by electroporation and thermal shock. T-test was used to evaluate the relationship and goodness of fit of the two strains. Given that $P > 0.05$ for two strains isolated from milk and cheese (0.559), there is no significant relationship between these two strains. As the tables show, higher number of colonies (352) was reported for electroporation, suggesting higher efficiency of electroporation compared to the thermal shock (76). As shown in Table 4, TE = 3.5×10^6 for the strain ATCC 14579 isolated from milk for the untreated cells and TE = 2.3×10^6 for the strain ATCC1098 isolated from cheese. As shown in Table 5, TE = 8.6×10^6 for the strain ATCC1098 isolated from cheese for the treated cells and TE = 7.5×10^6 for the strain ATCC14579 isolated from milk. The bacterial strains were treated by different concentrations of lysozyme, which result in different growth based on the concentrations used. In conclusion, the results show that both strains ATCC1098 and ATCC14579 receive more plasmid through electroporation.

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