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PROTOPLAST TRANSFORMATION OF TWO B. CEREUS STRAINSATCC14579 AND ATCC10987 USING PLASMID PIL253 BY ELECTROPORATION AND HEAT SHOCK

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Abstract

According to the studies conducted, Bacillus cereus is a gram-positive bacteria existing in soil and air. This type of bacteria is an opportunistic pathogen which can cause food poisoning. There isvery few number of bacterial transformation systems described. Favorable conditions are required for transformation of bacteria; because there are naturally a limited number of high quality bacteria, less bacterial transformation systemsare known. The purpose of this study is toexamineprotoplast of two strains of B. cereus extracted from milk and cheese using plasmid pIL253by electroporation andheat shock and compare the intake of each strain by plasmid. Finally, the results show that the strains of B. cereus ATCC14579 and ATCC1098 received theplasmid pIL253 by electroporation and heat shock and both ATCC1098 and ATCC14579 received more plasmid via electroporation.

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

Introduction

Bacterial transformation was first described by Greif (1928). Before identification of DNA, this descriptionwas as a genetic material. Greif observed that the combination of dead pathogenicpneumococcus cells andstrain of alive non-pathogenic cells (r) couldcause permanent transformation of rough stain (r) in the malignant cells (s). It was confirmed that transportation of DNA was taken by r-cells and it was high quality;therefore, it acquirednew genetic features. Members of at least 15 genera of bacteria are naturally high quality.The current knowledge about the natural process of DNA uptake has been studied in the literature. Very few bacterial transformation systems have been described. Favorable conditions are required for bacterialtransformation; because there are naturally a limited number of high quality bacteria, less bacterial transformation systemsare known. Bacillus cereus is a gram-positive bacteria existing in soil and air. This

type of bacteria is an opportunistic pathogen which can cause food poisoning (Schoeni&Wong, 2005; Jeßberger et al., 2014). Among groups of Bacillus cereus, B. cereus is very relevant to B. thuringiensis. B.mycoides and B.anthracisare the causal factors anthrax (Ash et al., 1991; Böhm et al., 2015). B. cereus is capable of producing toxins which cause gastroenteritis and several other virulence factorsincluding hemolysin, phospholipase, collagenase, protease, β-lactamase whichcontribute tonon-gastroenteritis infections(Washington et al., 2006). Genetic study is considered as an important target in a variety of biotechnological and microbiologic studies. Therefore, it is essential to transformDNA into the host cell. There are several methods for transformation; the most widely used ones are chemical method (heat shock) and electroporation. Due to the low efficiency of these two methods in strains of Bacillus, the protoplasts of two B. cereus strains, ATCC14579 and ATCC10987, were used in this study.Finally, these two strains were compared for two different methods of transformation.

Theoretical Background

Genetic engineering and various methods of gene transferallow the transfer of genes from any organism to another. Gene transfer methods are either direct or indirect(Mousavi, 2004). In indirect transfer,the gene is transferred by bacteria. British et al (1985) presented a gene transfer system for small and large plasmidsamongB. cereus, B. anthracis and B. thuringiensis bacteria basedonthe fertility factor B. thuringiensispX012. Raphael et al (1984) reported the transfer of small plasmids among these species using the phage B. cereus CP-51. Accordingly, Kohler and Thorne (1987) developed pLS20-mediated transfer of small plasmids. Another form of transfer is direct, whichis in fact the development of gene vectors in bacteria, often based on DNA transfer through bacterial intermediary hosts. In cases where the transfer of foreign DNA is not possible in this way, direct transfer methods are used based on the type and properties of the target tissue. In fact, direct gene transfer is a method in which the host cells receiveforeign DNA without intermediary hosts. Direct transfer involves: 1)Particle bombardment: this method was first reported by Sanford et al in 1987 and noted in various referencesas gene gun and biolistic method. The nucleic acid is poured on the particles of tungsten or gold with a diameter of 5 to half micron and sentrapidly (300-600m/s) into healthy tissuesby helium gas. Placing on a macrocarrier, microcarriers are propelledinto the gene gun chamber; they collide with a stopping screen (made of a metallic net) andpenetrate into the target tissue. In these circumstances, the particles are able to penetrate into the cell membrane without damaging it. 2) Electroporation: in this method, cells are exposed to high voltages for DNA transformation; in

fact, electric shockmakes small pores on the cell membrane temporarily;this makes the membrane permeable of the nucleic acid. 3) Microinjection and macroinjection: In the absence of natural DNA uptake, alternative means are required for transporting DNA into target cells for genetic works. A number of biological and physical changes occur in cells which are exposed to electricfields. These changes were first studied in 1972 by Neumann and Roshak. 4) Gene transfer via liposomes: one of the techniquesconsidered in the last two decades is to use liposomes in gene transfer. Liposomes are artificial membrane vesicles including a spherical bilayer phospholipid. By coating liposomes with nucleic acid, thecomplex, which is resistant to nuclease activity, is mixed by host cells. 5) Gene transfer using poly-cations: one of the methods of DNA transformation is touse long chain hydrophilic poly-cationssuch as polyethylene glycol (PEG), poly-L-lysine, or DEAE-dextran. Poly-cations can include metal ions such as calcium, sodium, potassium, rubidium, cesium, lithium and magnesium. This method can virtually be used in transformation of cell protoplasts for different species using very low amounts of DNA.

Plasmid is a small DNA molecule which exists in the cell separately from chromosome. Plasmids are replicated in the cytoplasm independent of genophore. Plasmids are usually in the form of a circular double-stranded DNA molecule (although there is a variety of linear plasmid).

A plasmid has an origin of replication (ori) where replication starts. Plasmids are naturally seen in bacteria, archaea (arcaebacteria), yeast and plants; however, they can be artificially transformed into animal cells. In nature, plasmids can provide organisms with better survival; for example, they can be carriers of antibiotic resistance genes. Plasmids are transferred from one to another bacterium via horizontal gene transfer. Plasmids are usually circular double-stranded DNA molecules.

Cosmid which was first introduced by Collins and Hohn in 1978is a synthetic plasmid containing the lambda cos sequence by which it can be well replaced in the head of the phage (Cos sequence + plasmid = cosmid). Cosmids aregene-cloning vectors which can carry 40kb cloned DNA and remain in E.coli in the form of plasmid. Cosmids have common properties of plasmid vectors and phage ʎ.

For example, a cosmid called as PLER-5 which is 6kb in length has two cos locations of ʎ phage cut byendonuclease I Sca, a multi-clone sequence with 6 unique locations for 6 different limiting enzymes (such as I EcoR and IIIHind), an origin of DNA replication and a tetracycline-resistant gene.

Literature Review

Recently, efforts has been made to increase the success rate of B. subtilis or a combination of osmoprotective factors and high electric fields. Similar studies have been conducted for pseudofirmus bacteria using osmolarity protection as well as treatment with glycine and high electrical field. These strategies have been tested for B. cereus group. The highest efficiency of electroporation is 103 cfcμg-1for B. cereus which is inadequate for many applications such as mutagenesis (Turjane et al., 2006). Turjane (2006) explained the electroporation protocol for B. cereus atcc14579 to evaluate the effect of changes in the growth cycle, electric field and the composition of amino acids (glycine and onine) to explain electric transformation of B. cereus atcc14579. An electric transformation was developed for B. cereus atcc14579. Efficiency of electroporation using stepwise growth culture and electric field was higher than 2 × 109 cfu μg-1 ml-1 with the plasmid DNA pC194. This type of process was tested in three other plasmids in various sizes with repetitive mechanisms and selective markers.Transformation efficiency(TE) varied from2×106 to 1×108 cfu μg-1 ml. Effects of twowall-attenuating factors on electroporation were also evaluated. Turjane reported the transformation rate as 103 times greater than the amount obtained forB. genus with similar plasmids and 106 times greater than the amount obtained by existing protocols forB. cereus. Tagavaisolated and identifiedflagellarof B. cereus ATCC14579; he found that flagellar filaments isolated from strain of B. cereus ATCC14579 contained 34, 32, and 31 proteins in similar proportions evaluated by band intensity on sodium dodecyl sulfate-polyacrylamide of electrophoresis gel. N-terminal sequences of the number of amino acid of these three strains of ATCC14579 protein was equal to the reduced sequences of three flagellin genes BC1657 and BC1658 and BC1659 in the total genome sequence. B. Strauss ATCC1479 is classified into serotypes T2 by flagellar serotype schemes of B. cereus strains. Flagellar filaments of the reference strain t2 contain two protein bands in 39 and 32kda; however, a protein band in 39dk was evaluated inflagellar filaments of the reference strain of serotype h1. Two mouse monoclonal antibody called a51 and a52 which recognize the flagellars 32 and 34kda as well as a flagellin 32kda reacted with B. cereus ATCC14579 strain and serotypes t2 in cell elisa and bacterial motility inhibition test. When using immunoelectron microscopy in conjunction with monoclonal antibodies 1a5 and 2a5, colloidal gold spheres were almost equal in flagellar filaments. Because the strain ATCC14579 and possibly serotype straint2 are unusual amongB. cereus strains when having multiple genes that encode flagellinsubunits, the unique possible mechanism may help the collection of multiple flagellin subunits in a filament. Keshtkar (2012) studied thedistribution and molecular properties of B. cereus

isolated from milk and dairy. This study determined the exposure to B. cereus and identified toxigenicgenes of B. cereus isolated from sterilized milk, pasteurized milk, yoghurt, cheese, cream and milk powder. PCR test results indicated the presence of genes hblA, nheA, nheB and nheC in 45 cases (97.82%) exposedto B. cereus and hbl B in 41 cases (89.13%). The genes bceTand entFM existed to a lesser extent in 20 cases (43.47%) and 15 cases (32.6%), respectively. The prevalence of B. cereus exposure was high in tested samples and mesophilicenterotoxigenicstrains which remain in heat-treated milk were the potential source of food contamination.

Materials and Methods

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

Methods

First, the bacteria B. cereus strains ATCC1098 (extracted from cheese) and ATCC14579 (extracted from milk) were cultured in a PAB medium at 37°C to OD600 = 1.7-2. Then, the protoplast was made by a method developed by Chang and Cohen.First, cells were cultured; the cell plate was collected by centrifugation. Then,the cell plates were solvedin the SMPP medium consisted of 2xSMM containing 1M sucrose, 0.04M malic acid and 0.04M 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, 10, 5 mg/ml) and mutanolysin (75U/ml) to obtain protoplast. The cells were collected from a 5200xg refrigerated centrifuge (4°C) for 5 minutes.

Measurement and Analysis

Transformation by heat shock and electroporation

Once the cells were cultured and new plates were taken, newly grown cells of 1.0M calcium chloride solution were used to rinse bacteria.Each rinsingstep required30min incubation on ice. After washing twice Kamptnt cells will be created, after Kamptnt stage and after the plasmid was placed adjacent cells Kamptnt put it at 42 degrees (heat shock) and then immediately put on ice. After two rinsing steps, competent cell was formed. Once the plasmid was placed near competent cells, it was heated to 42°C (heat shock) and placed on ice immediately. Next, 1ml SMPP medium was added and the

mixture was incubated at 37°C for 12 hours in 100rpm. Finally, the transformed bacterial plate was cultured in DM3 medium containing antibiotics for 48 hours at 37°C. For transformation by electroporation, cell plates of the made protoplast was collected after centrifugation and rinsed twice with electrotransform buffer. In total, 108 cells were used. Then, 120-microliterprotoplast was suspended in 2.5macroliter plasmid (1µg) and placed on ice for 5 minutes. Then, electroporation was done at 0.7kW, 400Ω and 25µF pulse. Next, 1ml SMPP medium was added and the mixture was incubated at 37°C for 12 hours in 100rpm. Finally, the transformed bacterial plate was cultured in DM3 medium containing antibiotics and kept for 48 hours at 37°C.

Transformation

Transformation efficiency (TE) is measured byTu method, written as follows:

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

Results

The results showed that different species have different growth properties; other results are related to receptor cells ATCC1098 treated with lysozyme:

Table-1: Changes in incubation time and concentration of lysozyme ATCC1098.

STDEV	Mean OD6002h	Lysozyme concentration (mg/ml)	Incubation time (min)
0.002	0.226	10	30
0.041	0.025	5	30
0.03	0.15	2.5	30
0.036	0.02	10	20
0.032	0.193	5	20
0.02	0.11	2.5	20
0.025	0.143	10	10
0.04	0.926	5	10
0.03	0.43	2.5	10

It was found that the highest growth of B. cereus ATCC 1098 (developing transformant) occurs when it is incubated by 10mg/l lysozyme for 20 minutes.

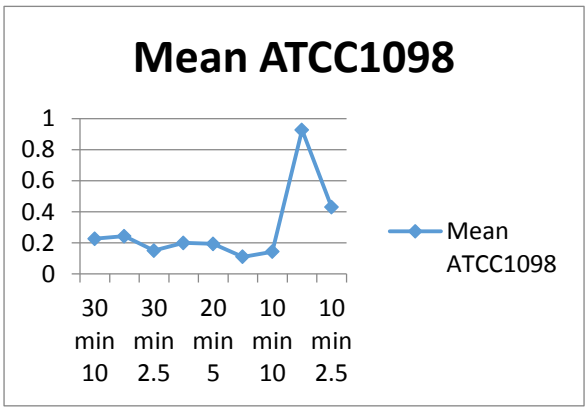

Figure 1: changes in incubation time and concentration of lysozyme ATCC1098.

Treatment ofreceptor cells ATCC14579 with lysozyme

Table-2: changes in incubation time and concentration of lysozyme ATCC14579.

STDEV	Mean OD6002h	Lysozyme concentration (mg/ml)	Incubation time (min)
0.003	0.204	10	30
0.025	0.213	5	30
0.02	0.13	2.5	30
0.017	0.16	10	20
0.015	0.146	5	20
0.005	0.086	2.5	20
0.499	0.676	10	10
0.03	0.93	5	10
0.03	0.416	2.5	10

It was found that the highest growth of B. cereus ATCC 14579 (developing transformant) occurs when it is incubated by 2.5mg/l lysozyme for 20 minutes.

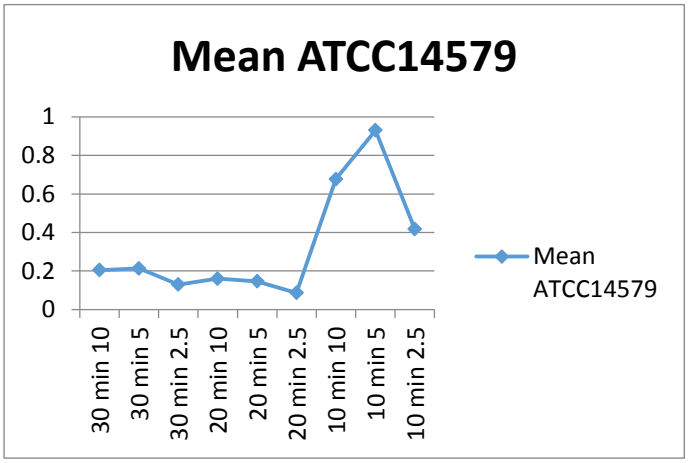

Figure 2: changes in incubation time and concentration of lysozyme ATCC14579.

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

Table -3: t-test to examine the relationship between strains.

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

T-test (t-student) is used to evaluate goodness of fitness or equality and inequality of sample mean and the population mean when the standard deviation is unknown. T-test results showedthat the mean of transformantis not fitted in two strains extracted from milk and cheese. Moreover, there is no significant relationship between these two strains.

Results of heat shock and electroporation

Results of heat shock and electroporation are listed in the table below as the number of colonies. According to results,mean = 76 and 352, and the standard deviation =4.5 and 6.11, respectively.

Table-4: number of colonies by heat shock and electroporation.

	Heat shock		Electroporation
1	Colony count		
	Mean	76	352
	Standard deviation	4.5	6.11
2	Plasmid		
	0.004µg/µl plasmid 200 µl $CaCl_2$ 250µl LB broth 1µl plasmid added to culture 100µl solution from the reaction tube spread on agar plate		

Transformation efficiency

Table-5: TE in the presence and absence of plasmid.

TE				
$CFE_{ATCC\ 1098}$	$CFE_{ATCC\ 14579}$	Untreated Cells	Plasmid	Receptor
-	-	$7.5 * 10^5$	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	

Discussion and conclusion

B. cereus is an aerobic gram-positive bacterium which is able to make spores. B. cereus contributes to the formation of microflora. Currently, some B. cereus strains are used as probiotic in livestock and poultry feed. For its ability to make spores and resistance to harsh environmental conditions, B. cereushas attracted attention of medical sciences, biology, veterinary medicine and industry. Genetic study is considered as an important goal in biotechnology and microbiology. Therefore, it is essential to transform DNA into the host cell. There are several methods for transformation; the most widely used ones are chemical method (heat shock) and electroporation. Due to the low efficiency of these two methods in Bacillus strains, the protoplasts of two B. cereus strains, ATCC14579 and ATCC10987, were used in this study. Finally, these two strains were compared for two different methods of transformation. The results showed that different strains have different growth properties, which means that optimum range in the 600nm wavelength was different for two strains to enter the exponential growth phase OD. According to the changes in incubation time and concentration of lysozyme ATCC1098, the highest growth of B. cereus ATCC 1098 occurs when it is incubated by 10mg/l lysozyme for 20 minutes. According to the changes in incubation time and concentration of lysozyme ATCC14579,the highest growth of B. cereus ATCC 14579 occurs when it is incubated by 2.5mg/l lysozyme for 20 minutes. T-test was used to evaluate goodness of fitness and the relationship between two strains. Given that the probability (0.559) was >0.05 for the strains

extracted from milk and cheese, there is no significant relationship between these two strains. Given the number of colonies calculated by heat shock and electroporation, higher number of colonies (352) are reported for electroporation which indicates the higher efficiency of this method than heat shock (76 colonies). TE was measured for protoplast of two B. cereus strains ATCC14579 and ATCC10987 using the plasmid pIL253 in the presence and absence of plasmid. TE was equal to 3.5×10^6 for the strain ATCC14579 isolated from milk, which is higher than that of the strain isolated from cheese (2.3×10^6). Finally, the results showed that the B. cereus strains ATCC1098 and ATCC14579 receive the plasmid pIL253 by electroporation and heat shock and both strains receive more plasmid by electroporation.

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