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AMELIORATE MANEUVER FOR TRANSFORMATION OF LACTOBACILLUS STRAINS BY ELECTROPORATION WITH IBDV-VP2 CHEMICALLY ENGINEERED EXPRESSION VECTOR

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ABSTRACT

Lactobacillus competent cells are considered important vehicles for electro-transform process and express exogenous DNA. Previously several complex protocols were used for electro-transformation of *lactobacillus* strains. In the preset study, we evaluated an ameliorate maneuver for the preparation of efficient competent cells of *lactobacillus*. The parameters like (i) washing buffers (ii) optical density 600nm (O.D 0.4-0.5) (iii) plasmid concentration i.e. 2µl-6µl (100ng/µl) (iv) Voltage 2.3-2.4 kv/cm were mainly focused. The high transformation efficiency was recorded in *Lactobacillus casie393* (9.9×10^2 and 2.4×10^2), *Lactobacillus pentosus* ($1.1\times10^3$and 3.1×10^2), *Lactobacillus plantarum* (1.2×10^3 and 3.7×10^2), with chemically engineered IBDV-vp2 expression plasmids viz., (i) pPG612-HCE-PgsA-vp2-rrnBT1T2, (ii) pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 by electroporation. Further confirmation of electro-transformation was analyzed by isolation, digestion and PCR. Hence this method proved simpler and efficient among previously employed methods in the preparation of *lactobacillus* competent cells. Consequently this procedure makes *lactobacillus* strains an excellent candidate for electro-transformation, more over could be used for electro-transform of other *lactobacillus* delivery vectors.

Key words: competent cell; IBDV-vp_2; electroporation; *lactobacillus*; transformation; ameliorate maneuver

INTRODUCTION

Since last few years, bacteria (e.g. gram-positive and gram-negative) are considered as an important carrier agent in the effective delivery of both DNA vaccine construct and vaccine antigens (Liljeqvist *et al.,* 1999; Gentschev *et al.,* 2001). Hence this technique makes it possible to administrate DNA vaccine through mucosal surface; in addition it helps to inject plasmid DNA directly into the professional antigen cells. Many authors reported both humoral and cellular responses against pathogens like HIV (Larisa *et al.*, 2004), and IBDV (Li *et al.,* 2006).

In molecular cloning, Plasmid transformation into the bacterial competent cells by using electroporation has been found an efficient technique (Ryu and Hartin, 1990). Later McCormac (1998) devised relatively a simpler procedure to produce competent cells viz., *Agrobacterium tunrefaciers* and *Agrobacterium rhizobium*. Whereas, previously used conventional methods to produce electro-competent cells were found time-consuming and laborious, furthermore these had least transformation efficiency (Enderle and Farwell, 1998; Zhiming *et al.,* 2005). Irrespective of these methods, Berthier *et al.,* (1996) reported electroporation technique for the transfer of plasmid DNA into lactic acid bacteria is considered more reliable, cost effective and efficient method (Berthier *et al.*, 1996).

However Electro-transformation is considered the most reliable and efficient tool for plasmid DNA uptake. It is the trendiest method for introducing exogenous plasmid DNA into lactic acid bacteria. Hence it seems to be an efficient technique for transferring plasmid DNA into lactic acid bacteria (LAB) (Berthier *et al.,* 1996). Since then, several electroporation methods have been developed to increase the transformation efficiency by the use of intact cells or combination of different cell wall weakening agents. These protocols mainly differ in the composition of washing agents, electroporation buffers and change in the electrical pulse according to the nature of DNA use for transformation (Ohse *et al.*, 1995; Xue *et al*., 1999; Ito and Nagane, 2001).

Electroporation is a mµlti-step process with several distinct phases (Weaver *et al.*, 1996). It mainly increases the permeability of cellular membranes, which allow the passage of larger and highly charged molecules like DNA (Neumann *et al*., 1982). However, transformation efficiency is strain-dependent and optimization requires improving transformation efficiency for a particular strain (Serror *et al.*, 2002). Whereas, the successful introduction of heterologous

plasmid DNA into LAB depends on the strains and application of plasmid vector (Bringel and Hubert, 1990; Thompson *et al.,* 1996). After the first successful application of electroporation in *streptococci* (Harlander and McKay, 1984), has initiated interest in the possibility of bacterial electro-transformation in LAB. Since then, a number of successful electrotransformation in *lactobacilli, lactococci* and other LAB have been documented (Scheirlinck *et al.,* 1989; Mercenier *et al.,* 1990; Gory *et al.,* 2001). The procedures and conditions of electrotransformation were found to fluctuate among the species and strains of LAB. Therefore, it is critical to standardize individual protocol for transformation efficiency improvement on this strain.

Previously a lot of literature is available on the preparation of *E. coli* competent cells with high transformation efficiency, however there is dearth of knowledge on simple, reliable and more efficient method to prepare high efficiency competent cell of *lactobacillus*. Moreover commercially prepared competent cells even much expensive and there is dire need for unconventional methods for the preparation of *lactobacillus* competent cells and electrotransformation. Therefore keeping in mind, the present study was designed to evaluate improved method of competent cell preparation of *lactobacillus* strains, focusing on the parameters, which standardize electro-transformation protocol for in vitro modified expression vector, ultimately enhance efficiency.

MATERIALS AND METHODS

Bacterial species and plasmid: In the present study three *lactobacillus* strains i.e. *Lactobacillus plantarum, Lactobacillus pentoses, Lactobacillus casei* 393 were used to make them competent. These expression systems were successfully electrotransformed with genetically engineered IBDV expression vectors viz., (i) pPG612-HCE-PgsA-vp2-rrnBT1T2 (ii) pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 containing VP2 gene, HCE promoter, T7g10 enhancer, PgsA anchor and cm resistant. These expression vectors were synthesized in preventive veterinary medicine laboratory, northeast agriculture university Harbin china.

Reagents and solutions for improved method

Washing buffer A: Washing buffer A was prepared in my laboratory by dilution of 1:1000 EPB into the water, set the pH at 7.4, autoclaved at 100°c. EPB is the mixture of Monosodium Phosphate $NaH_2PO_4.2H_2$ (.018g) and magnesium chloride $MgCl_2.6H_2O$ (0.04g) in 200ml H_2O. Further autoclaved and filtered.

Washing buffer B: Washing buffer B was prepared by 20% sucrose H_2O.

Extending MRS: Prepare Sterile 2% glycine MRS as an extending MRS for bacterial cµlture.

Recovery MRS: Sterile 20% sucrose MRS was used as recovery MRS.

Engineering of expression vector: IBDV vp2 gene was obtained from Veterinary Medicine College northeast agriculture university Harbin china .it was amplified by using polymerase chain reaction (PCR) with the specified pair of forward and reverse primer synthesized by 'BoShi Sheng Wu' Company. These forward (5'GAGCTCATGACGAACCTGCAAGAT3') and reverse (5'GTTAACCACCTCCATGAAGTACTCGCG 3') primers contained SacI and HpaI restriction sites respectively. Gene amplification was performed according to procedure documented by (Gang and Jing, 2007) with little modification in PCR system like: 95°c for 5 min, 20 cycles of 94°c for 1 min; 55°c for 1 min; 72°c for 90 s and 72°c for 10 min for final extension.. Vp2 was purified by digesting with restriction enzymes SacI and HpaI. It was ligated with pMD-18-Tsimple vector and transformed into TG1 and check for nucleotide sequence comparison of vp2 (Gene bank) with the public database by using program BLAST. The BLAST result was 99% of identity of vp2. This vp2 was purified and digested again with specified restriction enzymes and inserted into expression vector pPG612-HCE-PgsA-rrnBT1T2 (4954bp) to get pPG612-HCE-PgsA-vp2-rrnBT1T2 expression vector and also ligated T7g10 enhancer to pPG612-HCE-PgsA-vp2-rrnBT1T2 to get pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 expression vector. As a result got two expression vectors one with T7g10 enhancer and vp2 gene and other without T7g10 enhancer but with vp2 gene i.e. (i) pPG612-HCE-PgsA-vp2-rrnBT1T2 (ii) pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2.

Preparation of competent cells and bacterial electroporation:

Improved EPB (Electroporation Phosphate Buffer) method: Pick a single colony (2-3mm in diameter) from the growing plate that had been incubated for 16-20 hours at 37°c. The colony was transferred into 5ml MRS broth for 16-20 hours at 30°c without shaking in anaerobic condition. Starter culture (2ml) was used to incubate 100ml MRS extending medium for 2-3 hrs at 37°c until mid exponential phase ($O.D_{600}$ reach 0.5-0.6), transferred the culture flask on ice for 30 minutes. These cells were pelleted by centrifugation at 3500 rpm for 10 min at 4°c, then gently resuspend cells in one half volume (20ml) with ice-cooled washing buffer A (repeat this step for two times), later resuspend these cells with ice-cooled washing buffer B (20ml) at 4°c for 10 min (repeat this step for three times). Use 1ml washing buffer B to yield final competent cells suspension. Competent cells can be stored at -140°c for future use

Sucrose magnesium chloride method: *Lactobacillus* Competent cells preparation were performed as described previously (Ho, *et al*. 2005; Liu *et al*. 2012). A 2ml starter solution from 16-20 h *Lactobacillus* culture was inoculated into 100 ml MRS broth and incubated at 37°c without shaking. The cells were pelleted at OD 0.5-0.6 by centrifugation at 3000 g for 10 min at 4°c and washed twice with one half volumes (20ml), volume of ice-cold sucrose magnesium chloride electroporation buffer (SMEB) (250 mM sucrose, 1mM MgCl, 5mM sodium phosphate, pH 7.4). The cells were concentrated 100fold of original culture volume in ice-cold SMEB buffer.

Electroporation protocol use for this experiment: Electrotransformation of these competent cells were performed as described previously by Liu *et al.*, (2012) with some modification. Briefly, Took 200µl competent cells and add 6µl plasmid DNA (100ng/µl) and Incubate on ice for 20 min. Transferred to a pre-chilled cuvette (inter-electrode distance 1mm). The cuvette was connected parallel to 200 Ω resistor (pulse controller; Bio-Rad) generating peak field strength of 2.5kvcm^{-1}, time constant: 4-5ms. Immediately following the discharge, the suspension was diluted with 1ml recovery MRS, transfer into 15ml pre-chilled tube, add 2ml more recovery MRS to 3ml final volume. Incubate on ice for 5 minutes at 37°c for 2-3 hrs without shaking in anaerobic condition. Spread 300-500µl on the pre-warm antibiotic resistant MRS plate. Incubate plated at 37°c for 24-48 hrs in anaerobic condition.

RESULTS

Construction of expression vector: The 1287bp vp2 gene was successfully amplified by polymerase chain reaction (Fig. 3). Further it was cloned into a pMD-18-T simple vector, confirmed by sequence analysis. This vp2 gene ligated with an expression vector to generate pPG612-HCE-PgsA-vp2-rrnBT1T2 (Fig. 1) and also add enhancer to get pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 (Fig. 2), transformed into TG1 by the heat shock method. These plasmids were screened by PCR and single double digestion using restriction enzyme SacI and HpaI resulted in one vector fragment (pPG612-HCE-PgsA-vp2-rrnBT1T2, pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2) and two bands (vp2 band and vector band) (fig. 4.) PCR and restriction enzyme digestion analysis showed that recombinant expression plasmids pPG612-HCE-PgsA-vp2-rrnBT1T2 and pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 could be successfully constructed and transformed into TG1.

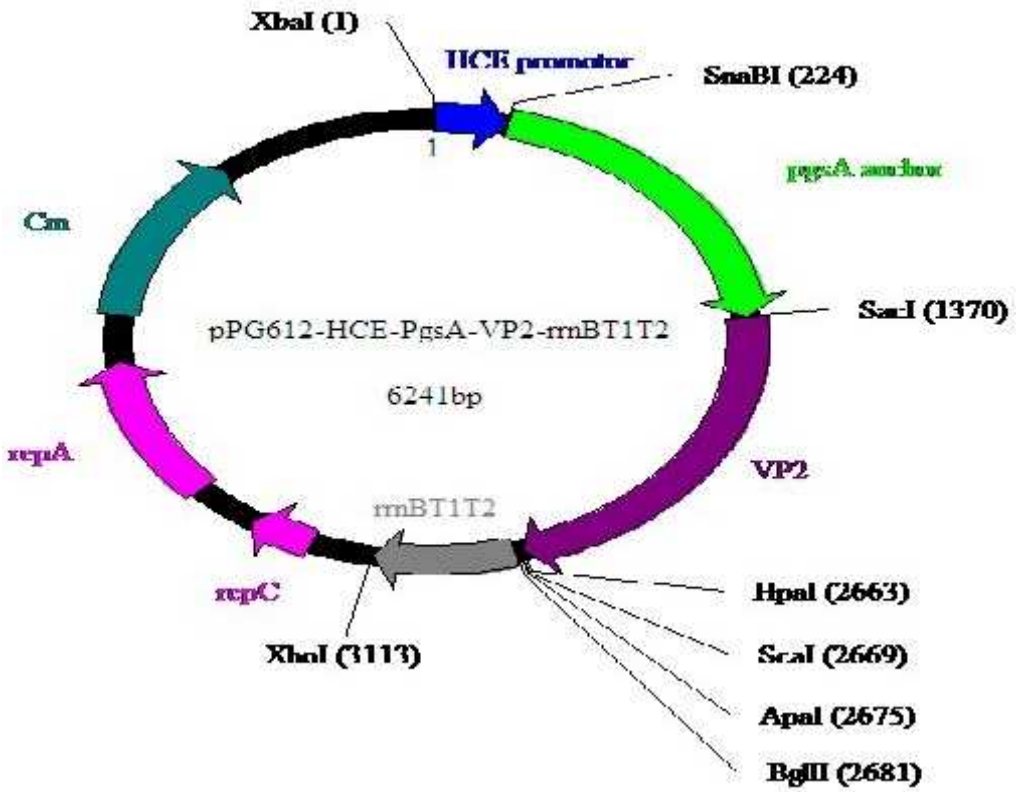

Fig. 1. Genetic Map for pPG612-HEC-pgsA-vp2-rrnBT1T2.The map shows different regions that are distinguished by different colours. This vector is cm resistant consist of 6241 base pairs (bp).

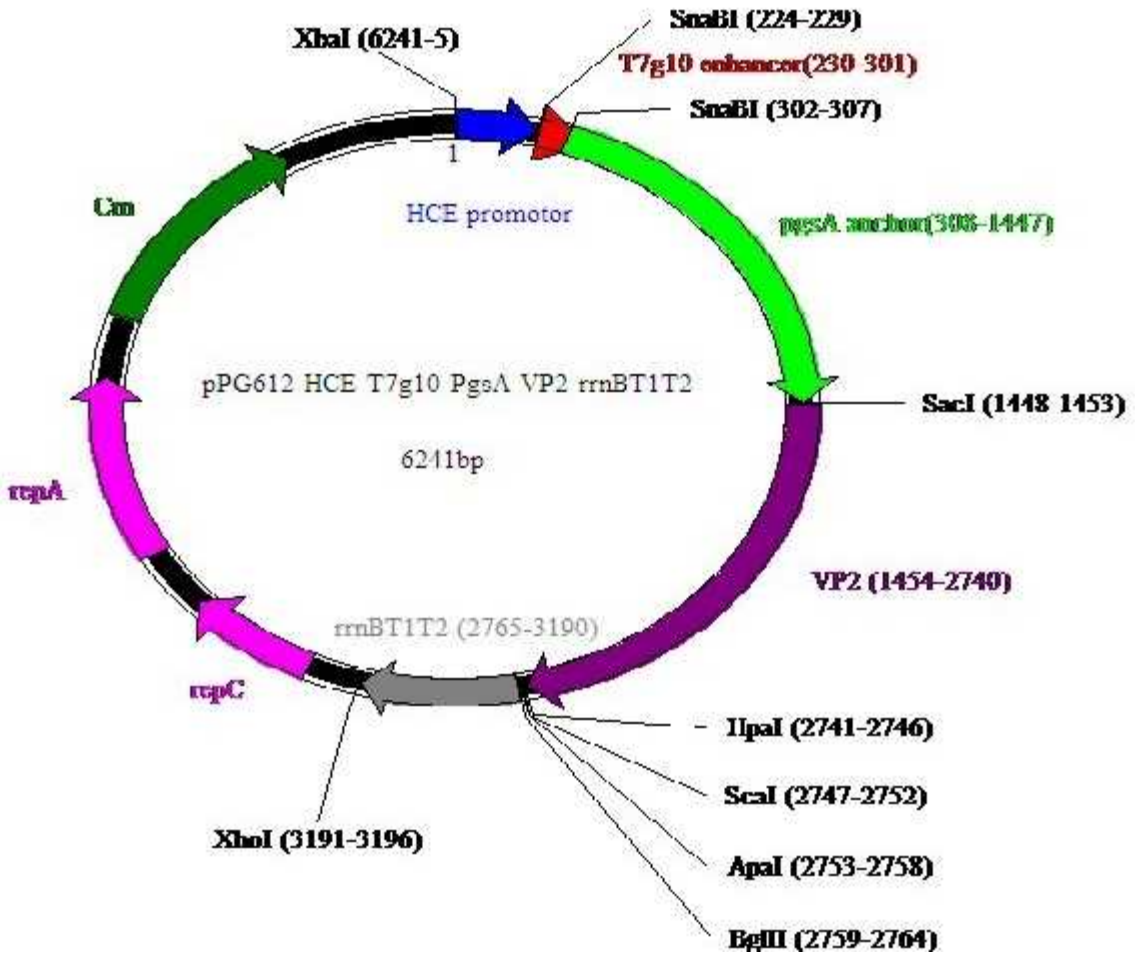

Fig. 2. Genetic Map for pPG612-HEC-T7g10-pgsA-vp2-rrnBT1T2. The map shows different regions that are distinguished by different colours. This vector is cm resistant consist of 6241 base pairs (bp).The map has an extra region of T7g10 enhancer which make it different from former vector.

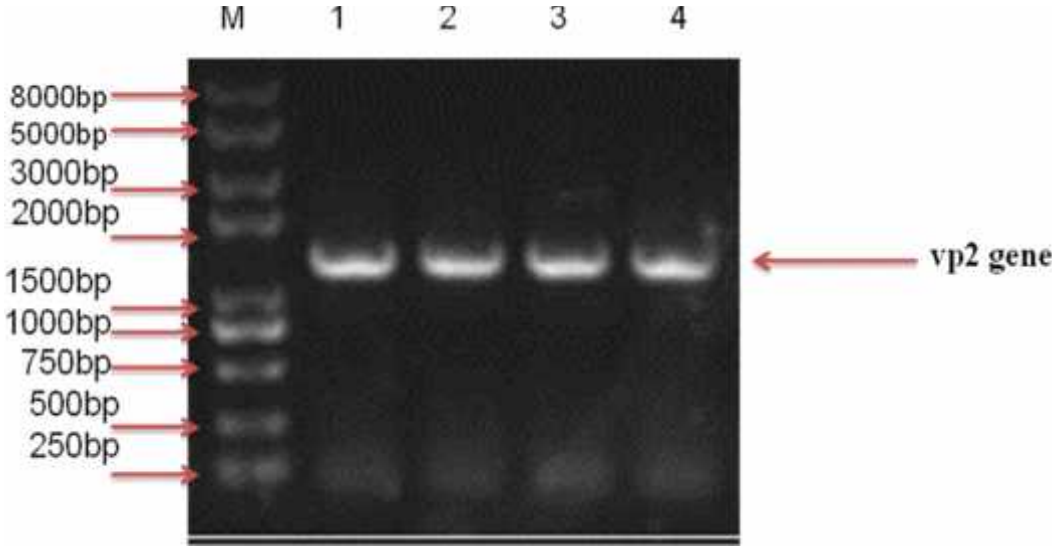

Fig. 3. PCR amplification of vp2 gene .Lane M Shows 8000bp Marker, Lane 1-4 shows PCR amplify vp2 gene

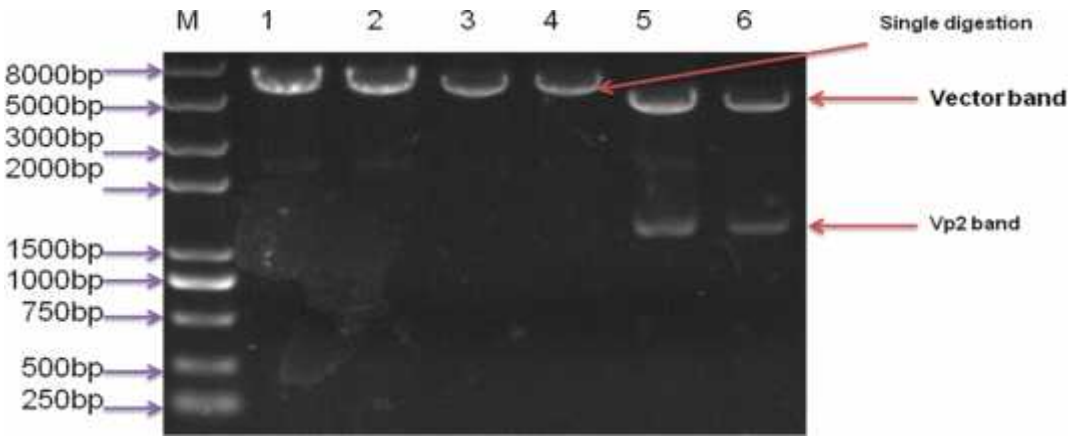

Fig.4. The Confirmation of recombinant plasmid by Single and double digestion. Lane M shows 8000bp Marker; Lane 1-2: Ppt612-T7-vp2/TG1, Ppt612-vp2/TG1 single digestion with sac I; Lane 3-4 single digestion with Hpa I; Lane 5-6 show double digestion of Ppt612-T7-vp2/TG1, Ppt612-vp2/TG1 respectively.

Electrotransformation of recombinant expression vector into *Lactobacillus* strains: Competent cells were prepared by using the method described in materials and methods. The electro transformed *L.casie*, *L.pentoses*, *L.plantarum* were used in vitro modified vectors i.e. pPG612-HCE-PgsA-vp2-rrnBT1T2, pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 isolated from *TG1*. We observed colonies on specific antibody resistant MRS plates. The respective *lactobacillus* transformant containing pPG612-HCE-PgsA-vp2-rrnBT1T2 and pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 plasmid DNA were extracted and subjected to restriction enzyme for digestion, PCR and sequencing was carried out for identification and confirmation of electro transformation. Moreover in both methods it was found that modification in following factors influenced electroporation i.e. washing buffer, O.D of bacterial growth, conc. of plasmid use for transfer and electric pulse.

Comparison of old and improved method: In the current study, three *lactobaccilus* strains were treated with both methods. high efficiency transformation of *L.casie* strains (9.9×10^2 to 2.2×10^2 and 2.4×10^2 to 3.0×10^1), *L.pentosus* (1.1×10^3 to 2.6×10^2 and 3.1×10^2 to 7.0×10^1), *L.plantarum* (1.2×10^3 to 4.9×10^3 and 3.7×10^2 to 1.2×10^2) (table 1) were achieved by improved and old method with chemically engineered IBDV-vp2 expression plasmids i) pPG612-HCE-PgsA-vp2-rrnBT1T2 (ii) pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 by electroporation respectively. We found that transformation efficiency by using improved method was high and sufficient for cloning needs. (Graph 1) Further analyses were carried out by plasmid isolation from these transformants strains and confirmed by PCR and digestion.

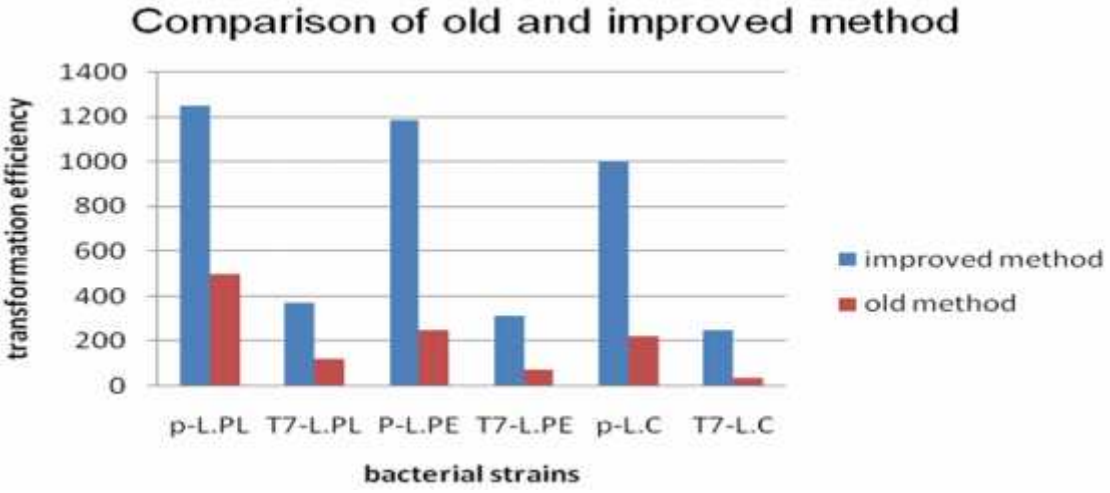

Fig 4. Effect of improved method on transformation efficiency of different bacterial strains. L.C (*L.casie*); L.PL (*L.plantarum*); L.PE (*L.pentosus*), P (pPG612-HEC-pgsA-vp2-rrnBT1T2), T7 (pPG612-HEC-T7g10-pgsA-vp2-rrnBT1T2).

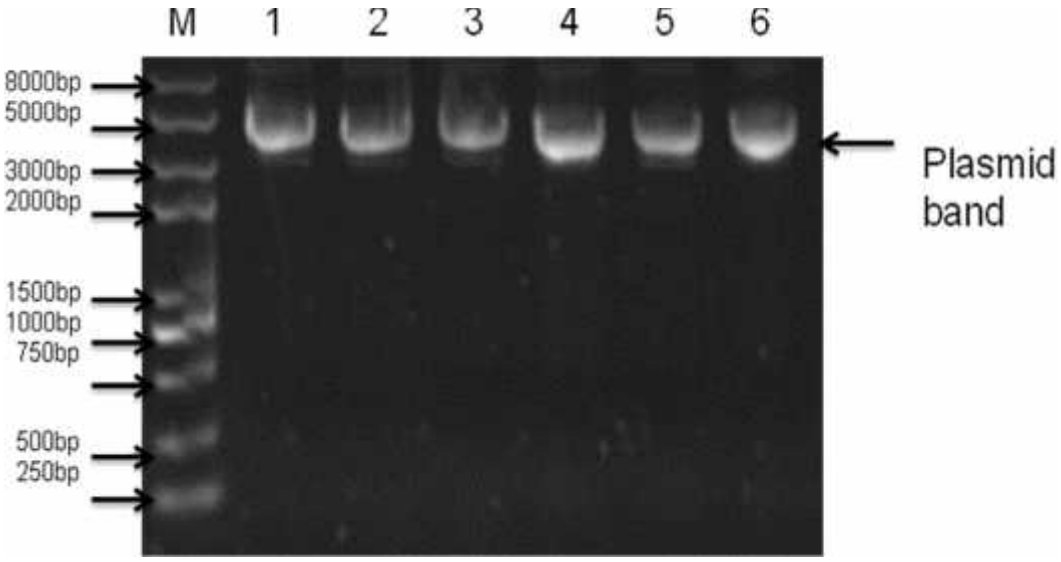

Fig.5: The Confirmation of trasformants by plasmid isolation. M: 8000bp Marker, 1-3: pPG612-HEC-T7g10-pgsA-vp2-rrnBT1T2 from *L.casie, L.pentosus* and *L.plantarum* , 4-6: pPG612-HEC-pgsA-vp2-rrnBT1T2 from *L.casie, L.pentosus* and *L.plantarum* repectively.

Table 1.Transformation efficiency of plasmid DNA to different lactic acid strains.

plasmid	strain	transformation efficiency	
		Improved method	**Old method**
pPG612-HCE-PgsA-vp2-rrnBT1T2	*L.plantarum*	1.2×10^3	4.9×10^2
pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2	*L.plantarum*	3.7×10^2	1.2×10^2
pPG612-HCE-PgsA-vp2-rrnBT1T2	*L.pentosus*	1.1×10^3	2.6×10^2
pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2	*L.pentosus*	3.1×10^2	7.0×10^1
pPG612-HCE-PgsA-vp2-rrnBT1T2	*L.casei*	9.9×10^2	2.2×10^2
pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2	*L.casei*	2.4×10^2	3.0×10^1

*Plasmid concentration and strains volume is constant for each transformation experiment. each volume was calcµlated as number of transformant per microgram of DNA

DISCUSSION

In recent years, many lactic acid bacterial (LAB) vectors have been constructed and used to express and deliver heterologous pathogen antigens (Maassen *et al.*, 1999; Scheppler *et al.*, 2002; Oliveira *et al.*, 2003; Ho *et al.*, 2005). Which are helpful to explore live bacterial vehicle vaccines for the prevention of infectious diseases especially IBDV. Bacteria that are capable to take up DNA are called "competent". In the present study, competent cells of *lactobacillus* strains viz., *L. casei, L. plantarum* and *L. pentoses* were successfully prepared by using simple and precise methods, which might be useful to prepare vaccines, moreover this method can also be used for other LAB strains. We made numerous attempts to electrotransform *Lactobacillus* strains with in vitro modified plasmid isolated from *E.coli* strain (JM109) by using previously described methods but no success (Liu *et al.*, 2012). Hence we developed a protocol (given in materials and methods) for electrotransformation. The designed protocol was found highly successful and we found transformant colonies of *lactobacillus casei, L. pentoses, L.plantarum* containing recombinant plasmid isolated from *E.coli* strain (JM109). For the purpose to make cell competent all the salt from the cell suspension must be removed by extensive washing. Although low salts buffer is usually used to remove these salts.

The most significant thing that the bacterial cells must be in their early logarithmic growth period, Ryu and Hartin (1990) has pointed out the significance of the log phase for transformation. The growth curves of three different *lactobacillus* strains are shown in Figure 4.

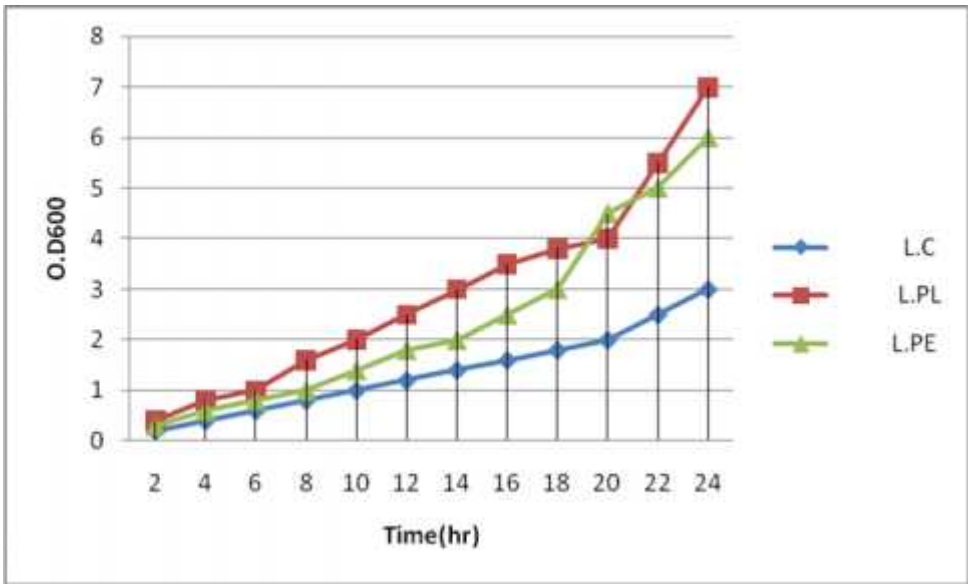

Fig4. O.D measurement in MRS medium. Bacterial growth was measured using OD_{600}.bacteria were transferred into MRS at a dilution of 1:100 and sample once every 2hr. the O.D was determined after 10 fold dilution. The horizontal axis indicates the cµlture time in hours; the vertical axis indicates O.D values. L.C (*L.casie*); L.PL (*L.plantarum*); L.PE (*L.pentosus*).

Competent cells prepared from $O.D_{600}$ of bacterial cµltures reached at 0.4 to 0.5 will have more efficiency and bacterial culture outside this optimal $O.D_{600}$ range will have low or no transformation capacity. Voltage is another important factor largely influences elctro-competent cells (Bringel *et al.,* 1990). In the present study 2.3-2.4 kv/cm range of voltage was found suitable for transformation process. Further Plasmid concentration also play important role in this process, hence various concentrations of plasmid were evaluated like 1µl plasmid DNA (100ng/µl), 10µl plasmid DNA (100ng/µl), 15µl plasmid DNA (100ng/µl), 20µl plasmid DNA (100ng/µl), however 6µl plasmid DNA (100ng/µl) was recorded most suitable for this process. Moreover we found that, if competent cells used soon after electroporation showed more efficiency than older one. These competent cells could also be preserved at -70 for up to 20 days, and at -40 for up to 5-7 days later they reduce their transformation efficiency (Zhiming *et al.,* 2005).

Using the protocol describes in materials and methods we prepare our competent cell and continue electroporation. During experiment we investigated that various factors i.e. washing buffer, O.D of bacterial growth, concentration of plasmid use for transfer, electric pulse influence electroporation. Our different attempts of electroporation showed that Voltage (2.3-2.4 kv/cm) were more suitable for the bacterial growth with optical density 600nm (0.4-0.5).In the current study, high efficiency transformation of *L.casie* (9.9×10^2 and 2.4×10^2), *L.pentosus* ($1.1\times10^3$and 3.1×10^2), *L. plantarum* (1.2×10^3 and 3.7×10^2), investigated with chemically engineered IBDV-vp2 expression plasmids; i) pPG612-HCE-PgsA-vp2-rrnBT1T2 (ii) pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 by electroporation.

Transgenic vectors pPG612-HCE-PgsA-vp2-rrnBT1T2, pPG612-HCE-T7g10-PgsA-vp2-rrnBT1T2 were constructed expressing vp2 protein of IBDV. VP2 protein is a host protective antigen. It induces virus-neutralizing antibodies that protect susceptible chickens from IBDV infection (Fahey *et al*., 1989; Macreadie *et al.,* 1990). In the current study we used these vectors for electro transformation into *lactobacillus* strains. Further studied are needed to assess the efficacy of vaccination in chicken with these delivery systems delivering recombinant expression plasmids encoding vp2 against IBDV.

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