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Study on the construction of recombined plasmid pMG36e-lacc1 and the electroporation of Lactobacillus buchneri

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Abstract. With a goal of obtaining the engineering probiotics that can produce both lactic acid and laccase, *Pleurotus eryngii* was selected as the test material. First, the laccase gene (Lacc1) was cloned by using RT-PCR (the length of which is 1596bp), and then, the gene was ligated to the food-grade vector pMG36e from *Lactobacillus buchneri*. The recombinant expression vector pMG36e-Lacc1 was constructed by transforming it into *Lactobacillus buchneri* via an electroporation method. The recombinant plasmid was constructed successfully as confirmed by gel electrophoresis. Then, the optimum conditions for electroporation were determined. The research revealed that when the electric field intensity is 1.75 kV with an SMRS medium recovery for 1.5 h, the electroporation translation efficiency reaches its maximum level.

Keywords: Laccase, *Lactobacillus buchneri*, expression vector system

1. Introduction

Silage is a kind of feed that uses fresh green plant as the raw material; the quality of the feed remains relatively stable by using *lactobacillus* fementation [1]. In fact, *lactobacillus* is the key factor in determining the quality of the feed. An ideal strain should be able to more quickly produce a greater amount of acid; however, this ideal strain has not yet been realized [2]. As the animal husbandry industry has rapidly developed, it becomes more necessary to improve silage quality through improving the silage's utilization rate [3].

However, laccase, a kind of copper polyphenol oxidase, may be key to improving silage's utilization rate [4]. Laccase possesses a strong ability to degrade lignin so that the degradation of cellulose can be fully achieved. In recent years, the function of laccase degradation has attracted greater attention. As molecular biology has further developed, a variety of laccase genes have been successfully cloned, and thus, their heterologous expression has been implemented [5] . In this paper, *Lactobacillus buchneri* was used as the experiment material. The optimal conditions were explored by using electroporation techniques to introduce genes into *Lactobacillus buchneri* with the intention of building a new *lactobacillus* that has both lactate and laccase metabolic products. With this new strain, the quality of the feed would be greatly enhanced.

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2. Materials and methods

2.1. Bacterial strains and plasmids

The *Lactobacillus buchneri* SYL from the silage that was used in the experiments is a strain that was isolated from Jia's Camp Ranch located in Inner Mongolia, China [6]. *Escherichia coli* TOP10 was purchased from the TIANGEN Corporation. The *P. eryngii* strains that were used in this study were collected from the Biotechnology Research Center of the Inner Mongolia Academy of Agricultural & Animal Husbandry Sciences. Plasmid pMG36e (from the Key Laboratory of Ion Beam Bioengineering at Inner Mongolia University) is 3.6 kb in size, which is useful for transferring genes between *Escherichia coli and the Lactobacillus* species. It contains the pWV01 origin of replication, a promoter p32, polyclonal loci (MCS), the repA gene, and an erythromycin resistance gene, which has been previously reported to be functional in a wide range of bacteria [7].

2.2. Measuring method

2.2.1. Lacc1 gene amplification

The *Lacc1* gene was amplified by RT-PCR as previously described by Dan Xue et al. [8].

2.2.2. Construction of recombinant plasmids pMG36e- Lacc1

Both the 1600bp fragment of recombinant pGM-T and vector pMG36e were digested with *Sph* I and *Sac* I (TaKaRa Biotechnology Co., Ltd. Dalian, China) and then ligated by T4 ligase (TakaRa Biotechnology Co., Ltd. Dalian, China). Next, the recombinant plasmid was introduced into *E. coli* TOP10, and then, the bacteria were spread onto LB agar plates containing erythromycin. After growth occurred, single colonies were picked and grown in LB liquid medium that contained erythromycin. The *Lacc1* gene was PCR amplified using specific primers. The recombinant plasmid pMG36e-Lacc1 was constructed.

2.2.3. The erythromycin susceptibility test and the preparation of electrocompetent cells

A *Lactobacillus buchneri* strain that had been preserved in the laboratory was activated according to reference [9]. The strains that produced the greatest acid amounts were selected. The competent cells were prepared, and the appropriate concentration of erythromycin was determined.

2.2.4. Electroporation conditions

Electroporation was carried out using the Eppendorf Electroporator 2510. Electroporation cuvettes (0.1 cm) were chilled on ice, and 50 μL of competent cells were mixed on ice with 5 μL of recombinant plasmids pMG36e- Lacc1. The mixture was mixed well and then allowed to sit on ice for 10 min. The mixture of cells and DNA was transferred to the bottom of cold 0.1-cm electroporation cuvettes and then electroporated at 0.0 kV, 1.5 kV, 1.75 kV, 2.0 kV, 2.25 kV, and 2.5 kV, respectively. Then, 950 μL of MRS or SMRS medium was immediately added after the pulse application. The entire cell suspension was transferred into a 1.5 mL tube and incubated at 37°C for 1.5 h, 2.0 h, and 2.5 h. Each experiment was repeated three times, and dilutions were spread ten times onto the MRS agar media containing 0.5 μg/mL erythromycin. The plates were grown at 37°C for 48 h. The transformation efficiency (cfu/μgDNA) was calculated by the following equation: *Transformation efficiency (cfu/μg DNA) = Appropriate dilutions × Colony count.*

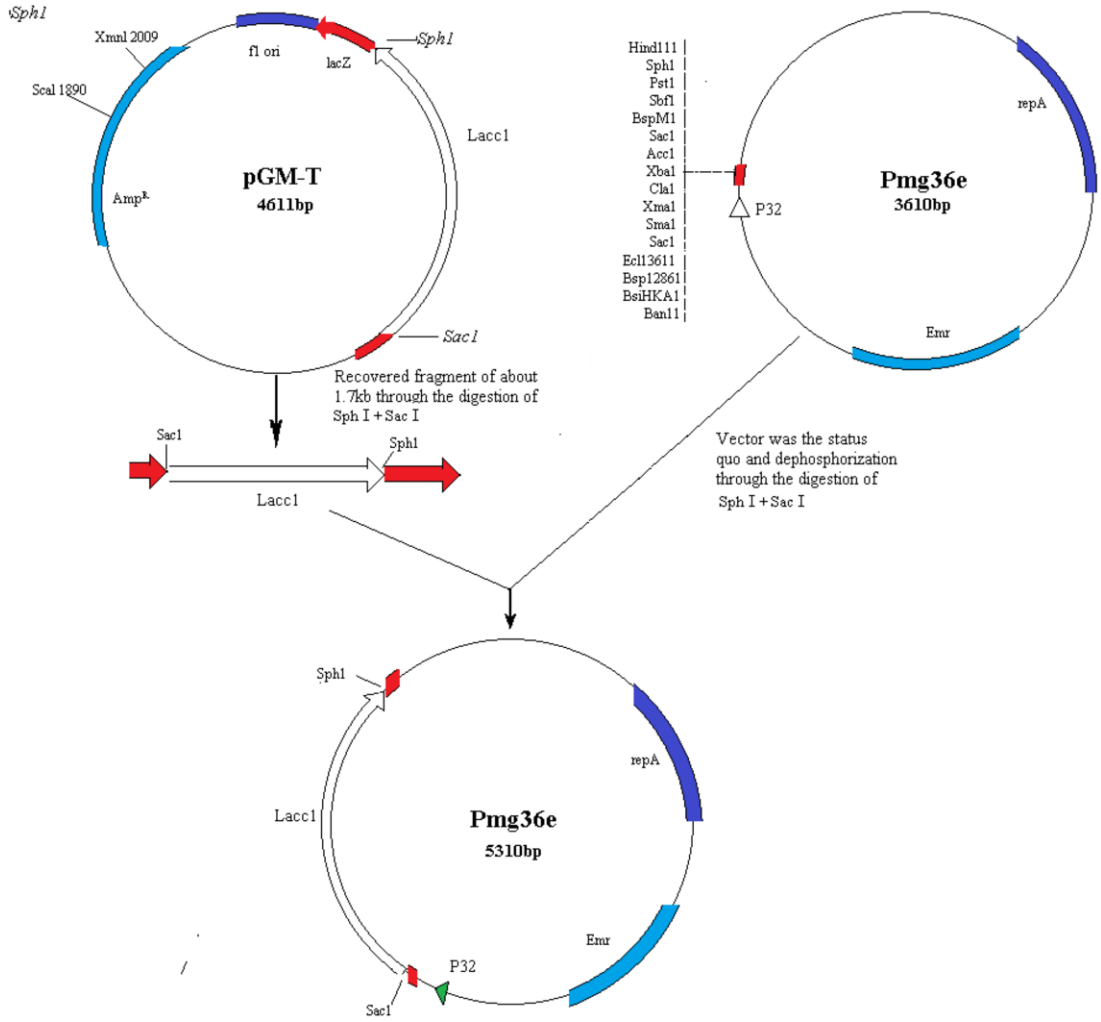

Fig. 1. Construction of recombinant expression vector pMG36e-Lacc1.

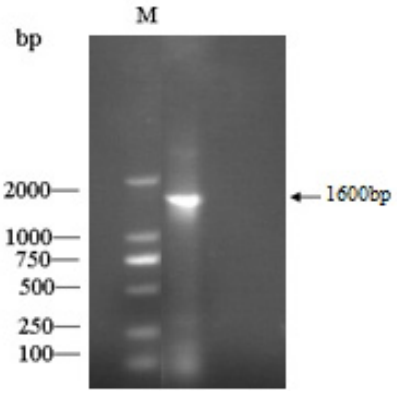

Fig. 2. Identification of recombinant plasmid pMG36e-Lacc1 by diagnostic PCR.

2.2.5. PCR identification of Lactobacillus's recombinant plasmid

The white colonies were picked directly from the MRS plates that contained erythromycin, and then, PCR was performed. The *Lacc1* gene was PCR amplified from the recombinant plasmid pMG36e-Lacc1. The PCR reaction system was as follows: 10 μL of 2Taq PCR Master Mix, 1 μL of primer P (10 pmol/μL), 1 μL of prime, Q (10pmol/μL), and 8μL of RNase-free water for a total volume of 20 μL. The PCR conditions were as follows: 93°C for 3 min; 35 cycles of 93°C for 15 s, 63°C for 30 s, and 68°C for 1.5 min; and then, indefinite at 4°C. Identification was confirmed using 1.0 % agarose electrophoresis. Since the correct 1600 bp fragment was amplified, the results confirmed that the recombinant plasmid was successfully transformed into *Lactobacillus Buchneri* through electroporation.

3. Results and Analysis

3.1. Cloning and sequencing of Lacc1

The PCR product was a DNA fragment of 1600 bp, the sequencing results verified that the PCR product was 1756 bp. Through DNAMAN analysis, the following data was obtained: the position of *Lacc1*'s complete open reading frame in 76 ~ 1672 bp (1596 bp in length), start codon ATG, and the termination codon TAG. The *Lacc1* gene open reading frame is composed of 1596 nucleotides, which encodes a polypeptide of 531 amino acids, as detailed in references [8].

3.2. Construction of recombinant expression vector of Lactobacillus

The recombinant pGM-T and vector pMG36e were digested with *Sph* I and *Sac* I after the gel recycling and then ligated using T4 DNA ligase, leading to the construction of pMG36e-Lacc1. The construction procedure is outlined in Figure 1. The full length of the 1600 bp fragment was amplified with specific primers as shown in Figure 2. Furthermore, by sequencing, the length of the 1596 bp segment was obtained, in accordance with the length of *Lacc1*. This confirmed that the recombinant expression plasmid pMG36e-Lacc1 has been successfully constructed.

3.3. The sensitivity of the Lactobacillus buchneri to erythromycin

When the erythromycin concentration is 0.5 μg/mL in the MRS medium, it can inhibit Lactobacillus growth. Accordingly, no colonies grew on a plate when the erythromycin concentration in the wells ranged from 0.5 μg/mL to 5 μg/mL. Therefore, a 0.5 μg/mL erythromycin concentration was selected as the minimum inhibitory concentration.

3.4. Electroporation conditions of Lactobacillus buchneri

As more research has been conducted on the molecular biology of Lactobacillus, there has been a series of cloning vectors and expression vectors based upon a *Lactobacillus* plasmid. The advantage of the *lactobacillus* expression system is favorable compared with that of the *E. coli* non- food -grade expression system. At present, protoplast transformation and electroporation are the main transformation methods for *Lactobacillus*. Protoplast transformation, however, has many unwanted constraints: it is time-consuming, has a low efficiency, and has a poor stability [10]. Thus, electroporation is widely used in *Lactobacillus* [11].

3.4.1. Effects of field strength on transformation efficiency

Different field strengths were selected for the electrotransformation. The recovered bacteria liquid was spread onto MRS agar media containing erythromycin. The varying transformation efficiency under different voltages and field strengths is shown in Figure 3.

As shown in Figure 3, the transformation efficiency initially increases as the electric field strength increases. When the field strength was 1.75 kV, the transformation efficiency reached its maximum: 2500 cfu/μg of DNA. However, as the field strength continued to increase to greater than 1.75 kV, the electrotransformation efficiency started to decrease.

3.4.2. Effects of recovery medium and time periods on transformation efficiency

In order to explore how the recovery medium and time periods would affect transformation efficiency, MRS (the *Lactobacillus* culture medium) and SMRS (MRS contains a certain amount of sugar) media were used for the recovery, and the culture condition was 37°C for 1.5 h, 2.0 h, and 2.5 h. The results are shown in Figure 4.

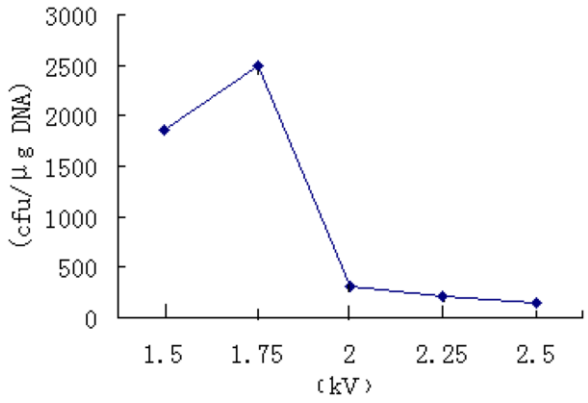

Fig. 3. Effects of electric field strength on electrotransformation efficiency.

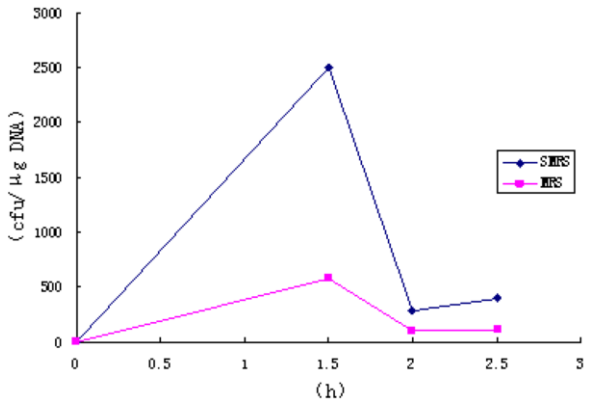

Fig. 4. Effects of recovery medium and time periods on transformation efficiency.

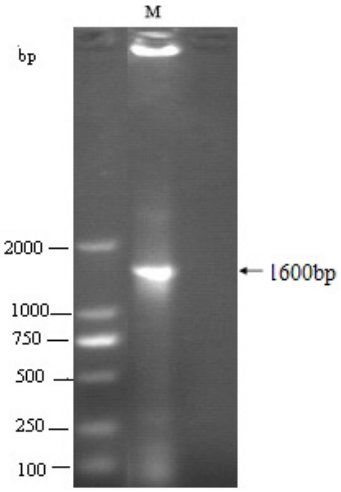

Fig. 5. The identification of recombinant plasmid in *Lactobacillus*.

As shown in Figure 4, an approximately fivefold increase in efficiency was observed when the SMRS medium was used for the recovery of the electroporated cells instead of the MRS medium. The highest transformation efficiency was obtained at a recovery time of 1.5 h, but further extending the incubation recovery results in a decline in electrical stimulation efficiency.

3.5. Detection of recombined Lactobacillus plasmid by PCR

The white colonies that had been selected from the MRS plates that contained erythromycin were detected by PCR, and the length of the 1600bp fragment was amplified. The colony PCR results confirmed that the recombinant expression plasmid pMG36e-Lacc1 has been successfully transformed into *Lactobacillus buchneri*. The PCR results are shown in Figure 5.

4. Discussion

Lignin and hemicellulose bind covalently to form complex natural polymers with an amorphous structure. The cellulose molecules are embedded in these polymers so that lignin cannot be digested by animals because there are no lignin degradation enzymes present in sheep and other animals. In order to make full use of straw, the lignin degradation must be solved [12]. Currently, white rot fungi is the main source of laccase [13]; at the same time, edible fungus is a green food, and the application of laccase that comes from edible fungi can ensure the safety of feed [14].

A study determined that the *Pleurotus eryngii* laccase enzyme activity is the highest, so it was selected as the test material for cloning the laccase gene. Additionally, *Lactobacillus* is a thicker cell wall, Gram-positive bacteria [15], so it is difficult for exogenous DNA to enter into the host cell. Thus, electroporation is commonly used to improve the transformation efficiency of *Lactobacillus*.

This paper primarily explored the optimum electroporation conditions of *Lactobacillus buchneri* that was isolated from silage, through the transformation of the laccase gene (*Lacc1*) connected with the food-grade vector pMG36e from *lactobacillus buchneri*. The recombinant expression vector pMG36e-Lacc1 was constructed by using the electroporation method to transform the vector into *lactobacillus buchneri*.

Then, conditions that affected the electroporation efficiency (electric field strength, recovery medium and time periods) were evaluated to determine the optimal conditions. For example, after exploring the effect that field strength had on transformation efficiency, data revealed that the transformation efficiency initially increased as the electric field strength increased, ultimately reaching a maximum efficiency of 2500 cfu/μg of DNA at 1.75 kV. However, after this point, as the field strength continued to increase, the electrotransformation efficiency started to decrease instead. Next, from exploring the effects of recovery medium and time periods on transformation efficiency, data indicated that there was an approximately fivefold increase in efficiency observed when the SMRS medium was used for the recovery of the electroporated cells instead of the MRS medium. When the recovery time was 1.5 h, the transformation efficiency reached its maximum; however, as the incubation time of recovery was further extended, the electrical stimulation efficiency began to decline. This is because with the extension culture by recovery medium (without antibiotics), *Lactobacillus* with a transfer plasmid and without the transfer plasmid were in the propagation process. The *Lactobacillus* without the transfer plasmid was breeding faster, and the plasmid-containing *Lactobacillus* perhaps lost the plasmid in the propagation process. Therefore, it is possible to conclude that that plasmid-containing *Lactobacillus* dwindled as time passed. This resulted in a decrease in the

number of colonies that grew on the MRS agar media that contained antibiotics. Then, as previously mentioned, as the incubation time of recovery was further extended, the electrical stimulation efficiency declined.

In this paper, the recombinant expression vector pMG36e-Lacc1 was successfully constructed by using an electroporation method to transform the plasmid into *Lactobacillus buchneri*. This will lay the foundation for the preparation of probiotic agents as well as obtain the engineering probiotics than can produce both lactic acid and laccase.

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