Optimization of *Agrobacterium*-mediated transformation in oyster mushroom (*Pleurotus ostreatus*) by vector containing human pro-insulin gene

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ABSTRACT

Transferring foreign genes into mushroom mediated by *Agrobacterium tumefaciens* is a standard technique in genetic engineering. Recombinant human insulin has been greatly used in the treatment of type I diabetes. The production of edible mushroom derived insulin should facilitate oral delivery. In this study we used the *Agrobacterium tumefaciens* mediated transformation method for the transfer and expression of the cholera toxin-B subunit (CTB) fused with human pro-insulin (Pins) in the edible oyster mushroom, *Pleurotus ostreatus*. The binary vector pCAMBIA1304 harboring CTB-Pins gene driven by the *P. ostreatus* gpd promoter was constructed and introduced into *P. ostreatus* via *Agrobacterium tumefaciens* mediated transformation. Optimization of gene transformation and tissue culture conditions is one of the most important issues in transgenic mushroom production. For attaining optimized conditions, the effects of co-cultivation conditions, *Agrobacterium* strains and the concentration of *Agrobacterium* cell suspensions were studied. Integration of CTB-Pins into mushroom genome was confirmed by PCR. The successful transformation to oyster mushroom suggests that the proposed modified transformation system is apparently useful for the production of human pro-insulin in these edible mushrooms.

Keywords: *Agrobacterium tumefaciens*, Diabetes, Gene transfer, *Pleurotus ostreatus*, Pro-insulin.

INTRODUCTION

Molecular farming is defined as the production of biopharmaceuticals and applied recombinant proteins in plants. The appearance of recombinant protein production technology has led to a universal excitement for the development of protein pharmaceuticals in the last two decades. These protein pharmaceuticals include functional regulators and supplements, enzymes, hormones, monoclonal and polyclonal antibodies and various vaccines (Meyers *et al*., 2008). Production of these valuable proteins via recombinant DNA technology is one of the most important areas in the pharmaceutical industry. Microorganisms, mammals, plants and insects have been developed as recombinant protein expression systems (Rigano and Walmsley, 2005). These bioreactors have been used widely for the production of desired foreign proteins. For the expression of a greater variety of high value proteins, it is essential to develop

other types of bioreactors. Previously, several expression systems such as yeast (Kjeldsen, 2000), *Escherichia coli* (Swartz, 2001) and plants (Mohebodini *et al*., 2009; Cunha *et al*., 2010; Tavizi *et al*., 2012) have been used to manufacture recombinant human pro-insulin. However, until now edible mushrooms have not been used as a bioreactor for the production of therapeutic proteins. To develop edible mushrooms as hosts for the production of protein-based pharmaceuticals such as pro-insulin, a DNA transformation system had to be developed.

A mushroom-based bio-factory offers several advantages over production systems. For example, their ability to carry out post-translational processing such as glycosylation, lack of endotoxins, storage cost and time efficient for marketing (Kim *et al*., 2010; Rathee *et al*., 2012). Moreover, this edible mushroom is a higher eukaryotic organism that can carry out protein-translational modification and therefore is a good bio-factory to produce pharmaceutical proteins (Kim *et al*., 2010). The first report on the transformation of edible mushrooms was demonstrated in 1998 (De Groot *et al*., 1998). Several transformation systems, such as polyethylene glycol (PEG)-mediated transformation (Li *et al*., 2006) and particle bombardment (Sunagawa and Magae, 2002) have been successfully employed for gene transfer into these hosts. Although these methods have been used for mushrooms, since the preparation of protoplasts for polyethylene glycol (PEG)-mediated transformation is difficult and time consuming for many mushrooms and gene transfer via particle bombardment and electroporation methods need expensive equipment. Therefore, *Agrobacterium tumefaciens*-mediated transformation (ATMT) could be a method of choice for gene transfer in fungi such as mushrooms. *A. tumefaciens* is capable of transferring a segment of its tumor inducing plasmid (T-DNA) into infected cells, and then this fragment integrates in the host genome and undergoes transcription (Tzfira *et al*., 2004). The ATMT method was demonstrated by De Groot *et al*. (1998) in spore for the first time and then has been applied to whole mycelia (Hanif *et al*., 2002; Combier *et al*., 2003) and fruitbodies (Chen *et al*., 2000; Cho *et al*., 2006; Wang *et al*., 2008; Kim *et al*., 2010).

Glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter is very efficient for gene expression in edible mushrooms including *Agaricus bisporus* (Chen *et al*., 2000), *Lentinus edodes* (Hirano *et al*., 2000) and *Pleurotus ostreatus* (Ding *et al*., 2011). Cholera toxin B subunit (CTB) is a pentameric subunit of Cholera toxin. CTB was used as an efficient carrier for genetically linked foreign proteins (Harakuni *et al*., 2005; Ruhlman *et al*., 2007). Therefore, in this study the pro-insulin conjugated to CTB driven by *P. ostreatus* gpd promoter was used for the oral administration of oyster mushroom-derived CTB-Pins fusion. Here we report the integration of CTB-Pins fusion gene into the oyster mushroom genome using *Agrobacterium tumefaciens*-mediated transformation method.

MATERIALS AND METHODS

Bacterial strains and culture maintenance

Agrobacterium strains including AGL1, EHA101, EHA105 and GV3101, which were conserved by the biotechnology laboratory of Tarbiat Modares University, were grown in LB (10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl) medium containing 50 µg/ml Kanamycin and 50 µg/ml Rifampicin (Hakim, Iran). Then bacterial clonies were used for the transformation of *P. ostreatus*. *Escherichia coli* DH5α was manipulated as described by Sambrook and Russell (2001).

Fungal material and culture conditions

The used fungus in this project was the Iranian *P. ostreatus* strain Iran1649c that was kindly provided by Dr. E. Mohammadi Goltapeh from Tarbiat Modares University of Iran. The vegetative mycelium was maintained at 25°C on Potato Dextrose Agar (PDA; Merck, Germany). For produce fruiting bodies, *P. ostreatus* was grown at 25°C and 80 percent moisture on wheat straw. For the selection of transformed cells, PDA medium was supplemented with Hygromycin (Duchefa, Netherlands).

Experiment to detect the suitable hygromycin concentration in the selection medium

To detect the suitable hygromycin concentration, mycelial plugs were cut from the colony edge with six mm diameter, and then transferred onto PDA plates with six different concentrations of hygromycin (0, 30, 40, 50, 60 and 70 mg/l) Separately, and incubated at 25°C for 14 days. Seven mycelia plugs were placed in each 90 mm Petri dish and replicated five times for each concentration.

Vector construction

The vector pCAMBIA1304 containing the hygromycin B selectable marker gene used in this study had been

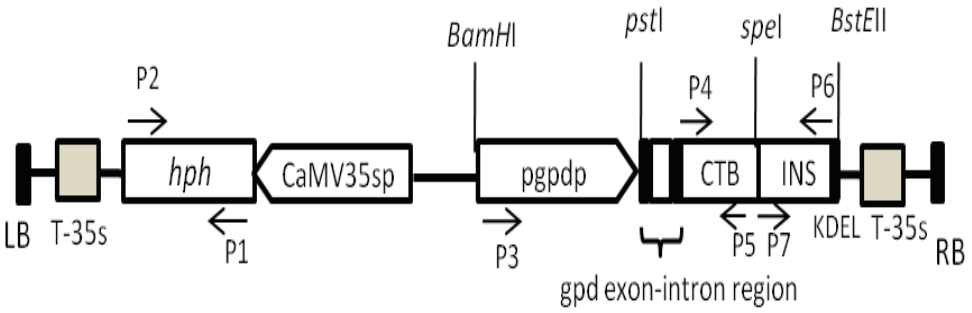

Figure 1. Modified binary vector PcambCTB-Pins containing gpd promoter. The expression cassette is 1644bp in size and consists of fusion gene encoding CTB-INS. The promoter consists of 913bp *P. ostreatus* glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter (Ppgpd) followed by the initial 100bp untranslated region of gpd which contains one intron. This cassette was inserted in upstream of Cauliflower Mosaic Virus terminator (T-35S), whereas hygromycin resistance (*hph*) gene is under the influence of Cauliflower Mosaic Virus 35S (CaMV 35S) promoter.

stored in our laboratory. For detection and analysis of heterologous gene expression in *P. ostreatus*, a new *Agrobacterium tumefaciens* binary vector, PcambCTB-Pins harboring cholera toxin B subunit (CTB) and human pro-insulin fusion gene under the control of the *P. ostreatus* gpd promoter (Ding *et al*., 2011), was constructed as follows. The pro-insulin gene was changed in order to include furin cleavage sites (RRKR) between the B-chain / C-peptide and the C-peptide / A-chain junctions and between the CTB / B-chain fusion site. Endoplasmic reticulum retention sequence (KDEL) (Wandelt *et al*., 1992), which can enhance the retention of proteins in the endoplasmic reticulum and thus leads to the increased stability and higher levels of accumulation of heterologous proteins, was also added to the C-terminal end of CTB-Pins chimeric gene (Figure 1). For the integration of this construct into pCAMBIA1304 binary vector, *Bam*HI and *Bst*EII restriction enzyme recognition sequences in the 5′ and 3′ ends were supplemented in the 5′ and 3′ ends, respectively. The sequence of this chimeric gene was codon optimized based on *P. ostreatus* codon preference and then an amplicon included *P. ostreatus* gpd promoter, CTB and human pro-insulin fusion gene was synthesized by Biobasic Company (Canada). Subsequently, this fusion construct was inserted into the pCAMBIA1304 vector backbone by digestion by *Bam*HI and *Bst*EII. This vector was introduced into *A. tumefaciens* strains through freeze and thaw method (Wise *et al*., 2006).

Agrobacterium-mediated transformation

Medium composition and bacteria preparation were carried out mainly as described by Chen *et al*. (2000). For transformation experiments, *Agrobacterium* strains were grown at 28°C in a minimal medium containing 50µg/ml kanamycin to an optical density of 0.6-0.8 at 600 nm. Bacterial cells were collected by centrifugation, suspended in an induction medium containing 200 µM acetosyringone, and incubated for an additional 3 h at room temperature with shaking at 100 rpm to induce virulence of *A. tumefaciens*. For AMT of the fruitbodies, gill tissue of oyster mushroom surface sterilized in 10% sodium hypochlorite solution for 1 min. The tissue pieces vacuum infiltrated by the suspension of induced bacteria. The evacuated tissue was transferred to a piece of sterile paper (0.2 µm pore size) overlaid on the co-cultivation medium and incubated for 3 days in various temperatures before being transferred to the selection medium for selecting putative transformants. Tissue pieces were transferred to selection medium containing 70 mg/ml hygromycin and maintained at room temperature at 25°C. Control mushrooms consisted of tissue pieces that were vacuum infiltrated by untransformed bacteria.

Experimental design for effective parameters on transformation efficiency

The transformation parameters including concentration of bacterial cells (0.6 and 0.8 at OD_{600}), bacterial strains (AGL1, EHA101, EHA105 and GV3101), co-cultivation temperatures (22, 25 and 28°C) were used to optimize gene transformation to oyster mushroom gill tissues using *Agrobacterium* strains harbouring the PcambCTB-Pins binary vector. The experiments were conducted as a completely randomized design (CRD) with three replications.

Table 1. Oligonucleotide primers used in PCR amplification of hygromycin (HPH), cholera toxin B subunit (CTB), human pro-insulin (INS) and CTB-INS fragments in the transgenic oyster mushroom.

Oligonucleotide name	Sequence (5′→3′)
P1 (HPH R)	ATGAAAAAGCCTGAACTCACC
P2 (HPH F)	CTATTTCTTTGCCCTCGGACG
P3 (Pgpdp F)	CGTTCGTGACTCGCAATATCAGTGC
P4 (CTB F)	ACCCCTCAAAAACATCACCG
P5 (CTB R)	GTTAGCCATCGAGATAGCAG
P6 (INS R)	TTAGAGCTCGTCCTTGTTGCAGTAG
P7 (INS F)	TTCGTCAACCAACACCTCTGC

DNA isolation and PCR analysis

Total genomic DNA was extracted from transformed mycelia and wild type mushrooms using LETS method (Romaine and Schlagnhaufer, 2006) and assessed by PCR analysis for the existence of *hph* gene and CTB-INS with primer pairs that listed in Table 1. Amplification conditions were, 5 min first denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C or 53°C, 70 s extension at 72°C and a final extension of 5 min at 72°C.

RESULTS AND DISCUSSION

Determination of the suitable hygromycin concentration

The use of proper concentration of antibiotic in the selection medium is essential in transformation experiments, in which the antibiotic serves as the selective agent that allows only transformed cells or fungi to survive. Hygromycin has been extensively used as a selective antibiotic in transformation experiments, mainly because several fungi transformation vectors possess hygromycin gene as the selectable marker (Chen *et al*., 2000; Hirano *et al*., 2000; Irie *et al*., 2001; Li *et al*., 2006; Scholtmeijer *et al*., 2001). Only transformed cells can grow in the presence of hygromycin. Mycelial plugs that were cut from the colony edge and transferred onto PDA plates with different concentrations of hygromycin. Colony growth was measured during a period of 14 days. The results are shown in Figure 2. Based on the results, mycelia grew very well without Hygromycin in PDA media. Mycelia growth significantly decreased on PDA media with increasing hygromycin concentration. The minimum lethal concentration to kill all the mycelial plugs in two weeks was 70 mg/l. Therefore, the concentration of 70 mg/l Hygromycin was used to select transgenic mushrooms in this research.

Agrobacterium*-mediated transformation of *P. ostreatus

The putative hygromycin-resistant transformed and untransformed *P. ostreatus* appeared after 14 days on the selection medium (Figure 3). The required time for the appearance of putative hygromycin-resistant colonies has been reported to be different in other fungi genera and strains. In *P. ostreatus* strain Pd739, the putative hygromycin-resistant transformant colonies were appeared after four days (Ding *et al*., 2011); where as, in *Agaricus bisporus* the appearance of transformants was reported after 9 to 14 days (Chen *et al*., 2000) and after 5 to 10 days in *Volvariella volvacea* (Wang *et al*., 2008) on the selection medium.

Effects of different factors on transformation efficiency

Due to the fact that the glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter has been successfully applied for the expression of heterologous genes in edible mushrooms (Chen *et al*., 2000; Ding *et al*., 2011; Maehara *et al*., 2010; Sharma and Kuhad, 2010), we have employed *P. ostreatus* gpd promoter for gene manipulation. Despite the fact that the basic principles involved in *Agrobacterium* mediated gene transfer into mushroom are the same for different mushroom species, the optimized condition for transformation depends on the species of the mushroom. Since the transfer of T-DNA from *Agrobacterium* to fungal cells is a complicated and time consuming process, the optimization of various parameters such as co-cultivation temperature, *Agrobacterium* strain and its concentration for efficient transformation is crucial. In this study, oyster mushroom gill tissue pieces were placed in each treatment combination and after three days of co-cultivation of *Agrobacterium* and gill tissue pieces (Chen *et al*., 2000) in various temperatures, they were transferred

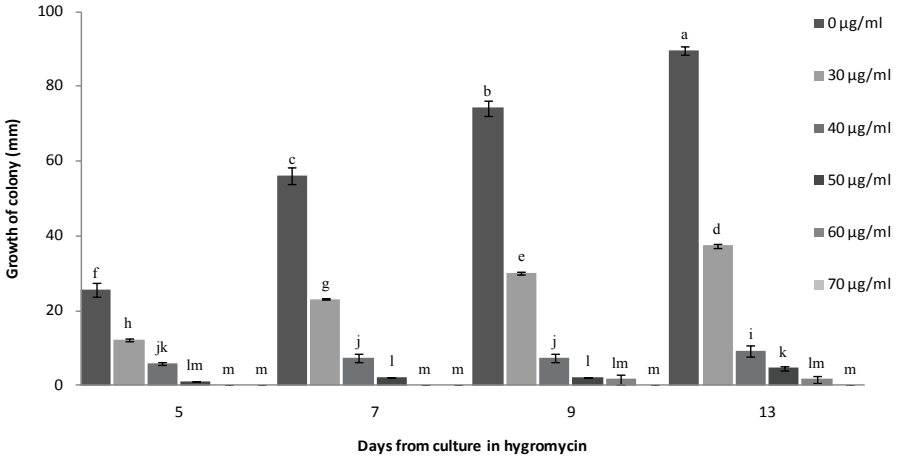

Figure 2. Mean comparisons of fungal colony growth with different concentrations of Hygromycin (0, 30, 40, 50, 60 and 70 mg/l) at 25°C for two weeks.

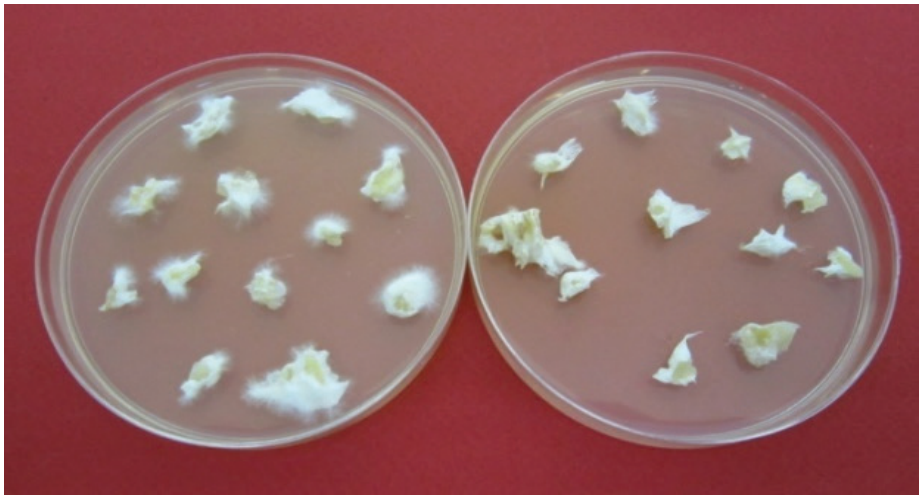

Figure 3. The appearance of putative hygromycin-resistant transformants of *P. ostreatus* after 14 days on selection medium with 70 mg/ml hygromycin. Pieces of gill tissues were co-cultivated with *A. tumefaciens* strain GV3101 (left) harbouring the vector PcambCTB-Pins containing the *P. ostreatus* gpd promoter and CTB-INS gene construct (right) without the vector PcambCTB-Pins as negative control.

on the selection medium supplemented with 70 mg/l hygromycin. The results are presented in (Tables 2-5), Because, no transformants were produced by *Agrobacterium* strains EHA105 and EHA10, they were left out in subsequent analyses. The results showed that transformation efficiency via *Agrobacterium* strain GV3101 (19.45%) was more effective and significantly higher than AGL1 strain (9.5%) (Table 2). This result was consistent with other studies that have been reported on this strain (Chen *et al*., 2000; Zhang *et al*., 2004; Ding *et al*., 2011). Maximum transformation efficiency was obtained in co-cultivation temperature 25°C (21.44%) (Table 3), bu it was 10% and 11.97% at 22°C and 28°C, respectively. Low efficiency transformation at higher co-cultivation temperature (28°C) could be associated with *A. tumefaciens* reduced activity to provide the

Table 2. Effect of *A. tumefaciens* strains on the transformation efficiency to oyster mushroom.

A. tumefaciens strain	Transformation efficiency (%)			
	Experiment 1	Experiment 2	Experiment 3	Mean
GV3101	19.88	18.19	20.28	19.45[a]
AGL1	10.45	9.22	8.82	9.50[b]

Table 3. Effect of *Agrobactrerioum and* gill tissues co-cultivation temperature on the transformation efficiency to oyster mushroom.

Temperature (˚C)	Transformation efficiency (%)			
	Experiment 1	Experiment 2	Experiment 3	Mean
22	11.43	8.91	9.69	10.01[c]
25	22.5	20.17	21.65	21.44[a]
28	11.56	12.04	12.31	11.97[b]

Table 4. Effect of *A. tumefaciens* optical density on the transformation efficiency to oyster mushroom.

Optical density (OD_{600}nm)	Transformation efficiency (%)			
	Experiment 1	Experiment 2	Experiment 3	Mean
0.6	12.95	11.64	12.12	12.24[b]
0.8	17.38	15.78	16.98	16.71[a]

virulence principle (Braun, 1947), furthermore at co-cultivation temperature 28°C , *P. ostreatus* mycelium grew significantly faster than *A. tumefaciens* during co-cultivation period of *Agrobacterium* and fungi. On the other hand, weak efficiency transformation at lower co-cultivation temperature (22°C) could be associated with the reduced growth rate of fungi mycelia and *Agrobacterium* cells. Our result was comparable with findings of Nyilasi *et al*. (2005). The highest number of hygromycin resistance fungi and high transformation efficiency was observed at OD_{600} 0.8 of *Agrobacterium* concentration (16.71%) than OD_{600} 0.6 (Table 4). The number of transformants obtained is dependent on the optimal combination of co-cultivation temperature and bacterial strain; these conditions need to be determined empirically for each combination of fungal strain and *Agrobacterium* strain. Also, during the optimization of *Agrobacterium* mediated transformation to *P. ostreatus*, it was noticed that temperature significantly influenced the number of transformants and background growth (Meyer, 2003). It is advisable to start with a systematic approach by testing a combination of different co-cultivation temperatures and different strains of *Agrobacterium* as a starting point for optimizing transformation frequencies of other fungi. Accordingly, in this research the interaction effect of *A. tumefaciens* strains × optical density × co-cultivation temperature showed that maximum efficiency (39.97%) was obtained with the combination of *Agrobacterium* strain GV3101 with OD_{600} 0.8 at co-cultivation temperature 25°C (Table 5). Our

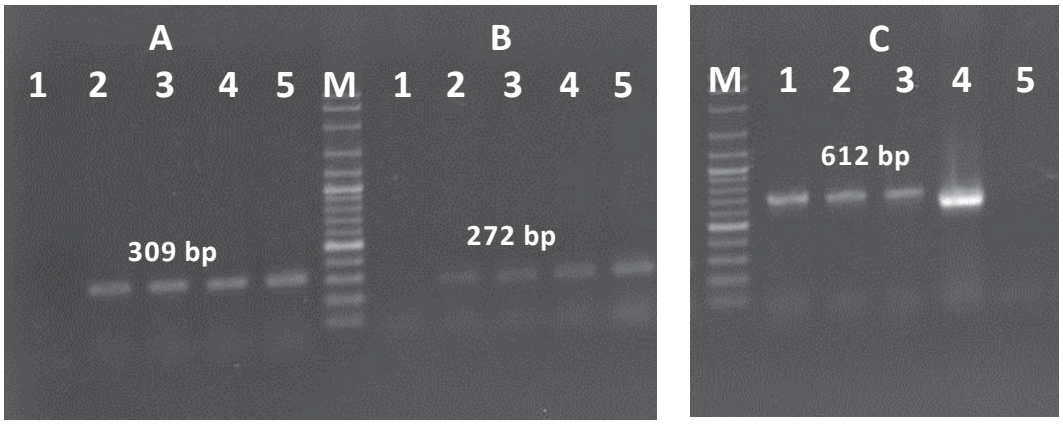

Figure 4. PCR amplification of **A:** cholera toxin B subunit (CTB) and **B:** human pro-insulin (INS) fragments in transgenic oyster mushroom. Lane 1: non-transgenic; Lanes 2-4: transgenic oyster mushroom and Lane 5: pCAMCTB-Pins plasmid (positive control). **C:** PCR amplification of CTB-INS fragment in transgenic *P. ostreatus*. Lanes 1-3: transgenic oyster mushroom; Lane 4: pCAMCTB-Pins plasmid; Lane 5: non-transgenic. M: DNA size marker (Fermentase, 100 bp).

Table 5. Mean comparison of *A. tumefaciens* strains × optical density × co-cultivation temperature interaction on transformation efficiency of oyster mushroom.

A. tumefaciens strain	GV3101						AGL1					
Optical density (OD_{600}nm)	0.6			0.8			0.6			0.8		
Temperature (˚C)	22	25	28	22	25	28	22	25	28	22	25	28
Transformation efficiency (%)	11.17^{d}	24.33^{b}	9.3^{d}	11.17^{d}	39.67^{a}	21.07^{c}	8.98^{d}	11.15^{d}	8.47^{d}	8.72^{d}	10.60^{d}	9.04^{d}

results showed that ATMT method (with transformation efficiency of about 40%) could be successfully applied to *P. ostreatus*. Comparing with previous reports on transformation of *P. ostreatus* via PEG-mediated transformation (Li *et al*., 2006), REMI method (Joh *et al*., 2003; Hatoh *et al*., 2013) and particle bombardment (Sunagawa and Magae, 2002), ATMT protocol exhibited more advantages, of which high transformation frequency was the most important one. Polymerase chain reaction (PCR) of transformants using specific primers of the human pro-insulin gene, cholera toxin B subunit and CTB-insulin fragments are shown in Figure 4. On the agarose gel transformed mushrooms displayed 272 bp (pro-insulin gene), 309 bp (CTB), 612 bp (CTB-insulin) fragments amplification products, which was not detected in non-transformed mushrooms (Figure 4).

CONCLUSION

Various hosts have been considered for the production of recombinant proteins, including mammalian cell lines. Some of these expression systems have a risk of contamination from potential human pathogens and none of them would be able to supply the demand for recombinant proteins required for full-scale treatment at an inexpensive cost. Moreover, the edible mushroom is a higher eukaryotic organism that can carry out protein-translational modification and therefore is a good biofactory to produce pharmaceutical proteins. Our result showed that by optimizing the conditions of transformation, even a recalcitrant crop like *P. ostreatus* can be transformed. Optimal conditions for transformation of oyster mushroom gill tissues were 3 days co-cultivation

of fungi with *Agrobacterium* strain GV3101 at 25°C. This fusion construct was inserted into the pCAMBIA1304 vector backbone. This vector was introduced into *A. tumefaciens* and successfully transferred to *P. ostreatus* using *Agrobacterium tumefaciens*-mediated transformation method. We report the introduction of the pro-insulin gene in edible mushroom.

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