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Design and Construction of Human mini-proinsulin gene, an Introduction for Transformation to Edible Button Mushroom (*Agaricus bisporus*)

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ABSTRACT: Diabetes is a serious, chronic disease that occurs either when the human body does not produce enough insulin, or when the body cannot effectively use the insulin it produces. The stages of protein metabolism in mushrooms are very similar to those in animal cells and plants. As a result, mushrooms have high potential to be employed in production of complex proteins such as secretion antibodies and oral vaccines. The importance of edible mushrooms is not only limited to their nutritional properties as delicious foodstuff, but also they play roles as food supplements, pharmaceuticals and bioremediation properties and their ability for production of recombinant proteins. Among of all known cultivated mushrooms, the white button mushroom, *Agaricus bisporus,* has the ability to make disulfide bonds and the post-translational changes, such as *glycosylation*, which is very similar to the human body. In the present study, firstly, the required primers were designed to amplify the *gpdII* gene promoter. The genomic DNA was then extracted from mycelium of the button mushroom. After performing PCR with the *pfu* polymerase, a 709-bp fragment which contains gpdII promoter was replicated. In addition, the synthesized mini proinsulin gene (PInk) was transfered to *E. coli* and kept and recultured with bacteria itself. Both plasmids contained the components of the promoter of the *gpdII* (PgpdII) and the mini-pronsulin gene (PInk) and also the double digested pBGgHg plasmid and the bonding reaction was performed to link PgpdII with PInk and entering the resulted part to the pBGgHg plasmid. The recombinant plasmid was designated as pCAMB-hmPInk for confirmation of map structure in digestion enzyme.

Keywords**:** *Diabetes, recombinant protein, recombinant plasmid, human mini proinsulin*

INTRODUCTION

Diabetes is one of the most serious and chronic disease in the world. It is estimated that there are more than 400 million people live with diabetes worldwide today. Type I diabetes (previously known as insulin-dependent, juvenile or childhood-onset diabetes) is characterized by deficient insulin production in the body. People with type 1 diabetes require daily administration of insulin to regulate the amount of glucose in their blood. This type of diabetes often affects children and adolescents, but aged people may also develop it. Type 2 diabetes (formerly called non-insulin-dependent or adult onset diabetes) results either from the body's ineffective use of insulin or the

body is unable to produce enough insulin [1]. Insulin is responsible for regulating blood sugar in the body. Human insulin protein is composed of two peptide chains referred to as the A chain and B chain (21 and 30 amino acids, respectively) and has a molecular weight of 5.8 kDa. A and B chains are linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. Although the amino acids sequence of insulin protein varies among species, some regions in the insulin molecule and amino acid positions are highly conserved. There are many similarities in the stages of protein metabolism between animal cells, plants, and fungi. These similarities are observed at all stages of protein synthesis, folding, protein assembly, and post-translational changes in the endoplasmic reticulum and Golgi apparatus. The result of these similarities is the representing of high potential plant cells and fungi to produce complex proteins such as secretory antibodies and oral vaccines [2].

Generally, fungi have important industrial and pharmaceutical uses. Fungi could play important roles in industry and medicine by secreting some of the most commonly used industrial enzymes (such as proteases) and primary and secondary metabolites (such as antibiotics) [3]. Among fungi, edible mushrooms are one of the commercial applications of microbial technologies for bioconvertion of agricultural waste into valuable food products [4]. According to statistics provided by the World Food and Agriculture Organization (FAO), global production of edible mushrooms in 2013 was about 9.9 million tons. Iran produced 8.7 thousand tons which has been placed seventh rank in the world for edible mushroom production after China (7 million tons), Italy (0.79 million tons), the United States (0.4 million tons), the Netherlands (0.32 million tons), Spain (0.14 million tons) and France (0.1 million tons) [5]. Edible white button mushroom (*A. bisporus*) is the most important edible mushroom in the world in terms of production and economic importance. In Iran, more than 98% of edible mushroom producers are engaged in producing edible button mushrooms which is very popular in Iran.

In addition to nutritional uses, recent studies have shown other applications of edible mushroom such as processed food and pharmaceuticals resource, bioremediation, and recombinant protein production. The white button mushroom has the ability to form disulfide bonds due to its eukaryotes, and post-translational modifications such as glycosylation occur similar to humans.

Fungi, like plant cells (in vitro culture) and animals, secretes protein into the culture medium, whereas does not occur in bacteria. The protein secretion system in fungi is more advanced than yeast and naturally secretes large amounts of protein, therefore in terms of the maintenance, the specific fungi strain which produced particular compound could keep for a long time. In addition, recombinant strain without particular compound requirement could keep in liquid nitrogen for longer time. Mushroom transformation has been performed using various samples at different life cycles(spores, mycelium and septum) with various methods, including *Agrobacterium tumefaciens-mediated* transformation (ATMT) [6], polyethylene glycol (PEG)-mediated protoplast transformation, restriction enzyme-mediated integration (REMI), electroporation, and ballistic bombardment**.** De groot et al. (1998) showed that the first transgenic studies in the fungi were carried out by Agrobacterium using *Aspergilus awamori* germinating basidiospores. Although the transgenic rate was not high in this experiment, it has been the first effective and repeatable experiment so far. Chen et al. (2000) have been successfully performed gene transformation into edible button mushroom using the ATMT method for the first time. By modifying the S35 promoter of pCAMBIA1300 plasmid and replacing the gpdII promoter of white button mushroom (glyceraldehyde-3-phosphate dehydrogenase), they constructed another plasmid called pBGgHg, which transformation and expression of the hygromycin phosphotransferase gene have been evaluated by a homolog promoter [7]. Isolation and extraction of recombinant pharmaceutical proteins is a highly sensitive and costly procedure, Thus edible mushroom as oral medications would be eliminated these steps and reduce production costs. The main objective of this study was to investigate the potential of expression and production of human mini-proinsulin in edible button mushroom. In order to achieve the objectives of current study, the design of a suitable structure for production of mini-proinsulin in edible button mushroom must be performed initially. The main purpose of the present study was to design and construct a plasmid structure containing the mini- proinsulin gene. The mini- proinsulin gene in this construct is controlled by the gpdII promoter of the white button edible mushroom (*A. bisporus*) whereas its sequence would be contained the KDEL regions at the end of the gene, the thrombin cleavage site and the Myc-Tag. In the following, recombinant plasmid containing mini- proinsulin gene expression cassette could be used for gene transformation to white button edible mushroom for further investigations.

Materials and methods

Preparation of liquid medium and culture of edible white button mushroom mycelium

To prepare one liter of liquid culture medium, 200 ml of compost extract was prepared by slight modification using Moloy (2004) method and then mixed with 20 ml of CYM (5X) storage solution [8,9]. For solid culture, a piece of mycelium is placed on a plate containing agar culture medium and kept at 25 ° C for 10 to 14 days. For mycelium culture in liquid culture medium, a piece of mycelium which has grown on agar culture medium

(2.5*2.5*2) is cut and gently placed on liquid medium. The culture vials were then incubated at 25±2 ° C for 14 days.

DNA extraction from edible mushroom and gpdII gene promoter isolation

Total DNA extraction of white button edible mushrooms was performed using GeneAll DNA Extraction Kit. After extraction to ensure the absence of RNA, RNase A enzyme from Thermo company at concentration of 50 µg / ml was used. Specific primers for the gpdII gene promoter were ordered from Cosmo genetic company to amplify the fragments containing the promoter. The amplified 709 bp fragment was then cloned into the TA vector using the TOPcloner TA core kit.

Bacteria and vectors used and storage conditions

In this study, Escherichia coli αDH5 and JM109 strains were used. E.coli was used as host for plasmid storage and replication. This bacteria was grown in LB medium with or without kanamycin (50 µg / ml).
The αDH5 strains of E.coli were obtained from the Laboratory of Industrial fungi biotechnology institute, ICCER and E.coli strain JM109 was provided from Laboratory of Biomolecular Systems at Gyeongsang National University – South Korea.

Edible mushroom strain and storage conditions

In this study, the modified M7219 strain of edible button mushroom from Mycelia Company was used. For culturing the desired edible mushroom mycelium, CEA medium was used and the plates were incubated at 25±2 ° C for 14 days.

Construction of plasmid structures

The pCAMB-hmPInk vector was designed and constructed based on the pBGgHg plasmid. The gpdII gene promoter was amplified from the edible button mushroom in a Enzynomics TOP clonerTM kit for clone fragment and the synthesized PInk gene was also cloned into the Puc57 vector and transformed into E.coli. Both plasmids containing the gpdII promoter and the PInK gene were digested by NotI and XbaI restriction enzymes. The ligation reaction was then achieved to bind the two digested fragments each other using Enzynomics T4 DNA Ligase. The gpdII gene promoter fragment was then cloned with the PInk gene and conserved as PPInk-TAV2 (Fig.1).

The plasmid pBGgHg and PPInk-TAV2 were binary digested by KpnI and XbaI (Fermentaz) restriction enzymes. The separated fragment was then inserted into the pBGgHg plasmid and kept as pkPInk-J83. Plasmid containing the gpdII promoter: To insert the gpdII gene and the proinsulin gene into the pBGgHg plasmid, the gpdII gene promoter was amplified by polymerase chain reaction using Pfu enzyme. At the beginning and end of the gpdII promoter, sequences were arranged for NotI and XbaI enzymes, respectively. The amplified fragment was then cleaved into pTOP V2 plasmid by restriction enzyme. Plasmid containing the proinsulin gene: the synthesized proinsulin gene with restriction enzyme sites at NotI and KpnI has been placed in the end of synthesized gene and pTOP V2 plasmid.

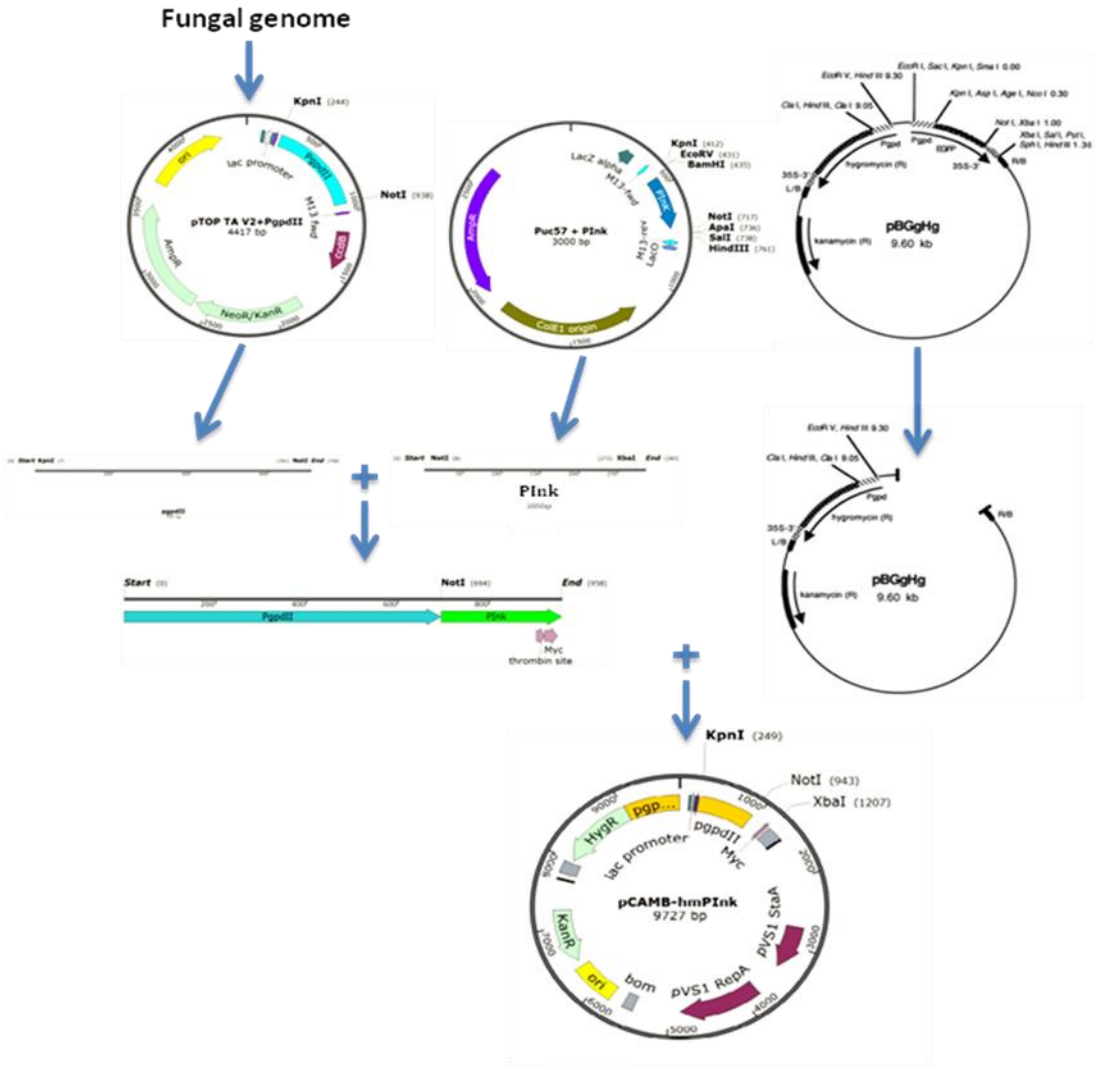

Figure 1: Steps of construction Recombinant Plasmid pCAMB-hmPInk

Result and Discussion

Recombinant protein production has been one of the most important accomplishment in biotechnology and genetic engineering in recent years. Currently the demand for recombinant human proteins for the treatment and diagnosis of diseases increase dramatically which one of the most important one is diabetes. Approximately 0.8% of the world's populations suffer from insulin-dependent diabetes, and it has been anticipated that by 2025, the number would be reached nearly 300 million. The high demand of this hormone necessitates the development of inexpensive, cost efficiency and high production capacity methods in the future [10].

DNA double-strand breaks in the eukaryotic genome are mainly repaired by homologous recombination (HR), single-strand annealing, microhomology-mediated end-joining, and non-homologous end-joining (NHEJ). Introduction of a foreign gene into living cells and deletion of a target gene from genomic DNA are essential steps for better understanding cell biology at the molecular level. Nevertheless, mushroom transformation has not been freely accessible partly due to the dikaryotic nature of mushroom mycelia. More importantly, mushroom cells have

their own limitations as good hosts for foreign DNA since HR is suppressed in filamentous fungi, whereas NHEJ is the major repair system against DSBs. With NHEJ, integration of foreign DNA has to be random, which makes it difficult to target a specific gene. Therefore, suppression of NHEJ by downregulation or knockout of NHEJ components is essential and conceivably a prerequisite. Filamentous growth with connected cells is another obstacle since it prevents isolation of single cells from certain experimental treatments and thus causes difficulties in the selection of independent cells with a desirable phenotype. Use of germinating spores, protoplasts, and finely broken mycelia with regenerated apical tips may avoid this problem. Generation of a variety of auxotrophic mutant strains is also needed to make mushrooms as an efficient host as yeast [11].

Oral insulin administration is one of the new efforts, but the major challenge in using insulin orally need to protect it from enzymatic digestion and absorption in the intestinal tissue. Methods such as insulin encapsulation using nanoparticles and insulin use along with anti-protease compounds have been introduced to overcome this problem [12]. There have also been attempted to express insulin in plant cells. Edible mushroom which have high potential in nutritional and pharmaceutical properties could be considered as oral medications. This is not only desirable for pharmaceutical companies but, if it achieved successfully, it would open the new path to the supply of low-cost medicines in under developed countries. Finally, the expression of recombinant insulin protein production has been examined. Recombinant human mini-proinsulin is a novel insulin precursor with a shortened C-peptide chain which could be easily converted by enzymatic cleavage into human insulin [13]. Recently, a number of mini proinsulins with various mini C-peptides were produced to increase the folding efficiency of the insulin precursor and the production yield of insulin [1]. Ahmad et al. (2015) have been studied a gene coding for a novel pre-miniproinsulin analogue with a potential industrial application for recombinant human insulin production was synthesized using DNA technology, cloned in a suitable expression vector and expressed in a selected Escherichia coli strain.

Ni et al.(2007) have been studied oral route of mini-proinsulin-expressing *Ganoderma lucidum* which decreased blood glucose level in streptozocin-induced diabetic rats. A new mini-proinsulin gene was constructed and was transformed into *G. lucidum* by the Agrobacterium mediated method. Ni et al.(2007) hypothesized that the natural structure of the cell wall and the endoplasmic reticulum in *Ganoderma lucidum* should improve the resistance of insulin produced in these cells against digestion and introduced a new delivery system for oral insulin [12]

PInk gene with 285 nucleotide consists of nucleotide sequences of chains A and B using recommended codons for edible button mushrooms, sequences between two chains [13], KDEL sequences [14], A cleavage site for the thrombin [15] and Myc tag sequences [16] were synthesized. The use of significant codons is reported for all organisms. The preferential use of codons in an existing genome is more intense for genes with higher expression. Studies on genes that have high expression in edible button mushroom (such as laccglycerqldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase) provide beneficial information for selecting preferred codons. It has been principally observed that the edible button mushroom prefers the position of the third codon with C or T nucleotides. The gene was ordered from Cosmo Genetic Company and was inserted into the puc57 plasmid. The use of the KDEL sequence in gene transmission and the effect of this sequence in enhancing the expression of recombinant proteins has been demonstrated. After synthesis of the initial mRNA sequences, proteins containing the KDEL sequence are transferred to the Golgi apparatus by entering the endoplasmic reticulum. The receptors in the Golgi apparatus identify the KDEL signal sequence and form a bond to its protein. Following, proteins containing KDEL is restored to the endoplasmic reticulum by COPI-coated vesicles. Myc-Tag is a powerful tool for protein site detection, ELISA testing as well as protein purification. The Myc-Tag amino acid sequence is N-EQKLISEEDL-C. This tag contains 10 amino acids and molecular weight is about 1202 Daltons. In current study, a gene construct containing human mini-proinsulin gene was recorded in NCBI database under the code Pink-J83 and gene code bank KX161700.1. The gene construct, previously optimized for codon preference of edible button mushroom, was synthesized by Cosmogene tech company and delivered in pUC57plasmid. Both TAV2 and pBGgHg plasmids carrying pgpdII + PInk fragment were subjected to double enzymatic digestion with two KpnI and XbaI restriction enzymes. Enzymatic digestion of the pBGgHg plasmid with the restriction enzymes resulted in the removal of the 1000 bp fragment. Then the isolated fragments of both TAV2 plasmid (pgpdII + PInk) and linear pBGgHg plasmid were purified from agarose gel. To achieve the binding reaction, the purified fragments of agarose gel were mixed with 3: 1 pgpdII + Pink to pBGgHg plasmid in the presence of T4 DNA ligase with the corresponding buffer and the pgpdII + Pink fragment was ligated to pBGgHg plasmid [17]. Finally, the 968 bp fragment was cloned into the pBGgHg plasmid and a new recombinant plasmid was designated as pCAMB-hmPInk. The pBGgHg plasmid is constructed from the pCAMBIA1300 plasmid and designed by Chen et al. (2000) and contains the hph selective gene and the EGFP marker gene (Fig.2).

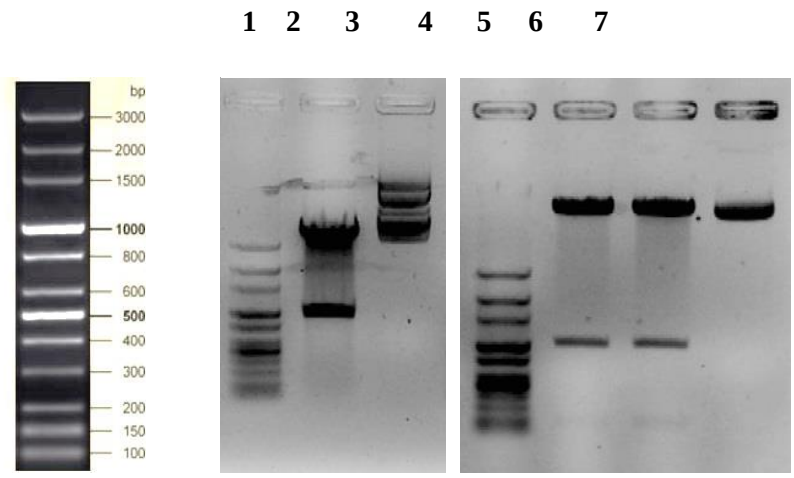

Figure 2: Enzymatic Double Digestion Electrophoresis of Two DNA plasmid TAV2 + Plasmids (pgpdII + PInk) (1: 3kb DNA Ladder, 2: TAV2 + Plasmid (pgpdII + PInk) double Enzyme Digestion with Two restriction Enzymes KpnI and XbaI, 3: TAV2 +(pgpdII+PInk) and pBGgHg Plasmid (4: 3kb DNA ladder, 5 and 6: double digestion of pBGgHg plasmid, 7: pBGgHg plasmid).
(1% agarose, V100 voltage, TAE 1X buffer and 3kb (Jena Bioscience GmbH) Mid Range DNA Ladder)

The recombinant pCAMB-hmPInk plasmid was then transferred to E.coli susceptible JM109 strain for storage and transgenic colonies were grown on selective medium containing kanamycin (50µg / ml). To confirm gene cloning in pCAMB-hmPInk recombinant vector, Colony-PCR assay was achieved on cultured colonies in media containing kanamycin (50µg / ml). Colony PCR has been carried out using PPInk1200 primers and the presence of bp1246 fragment in some of them was confirmed (Fig.3).

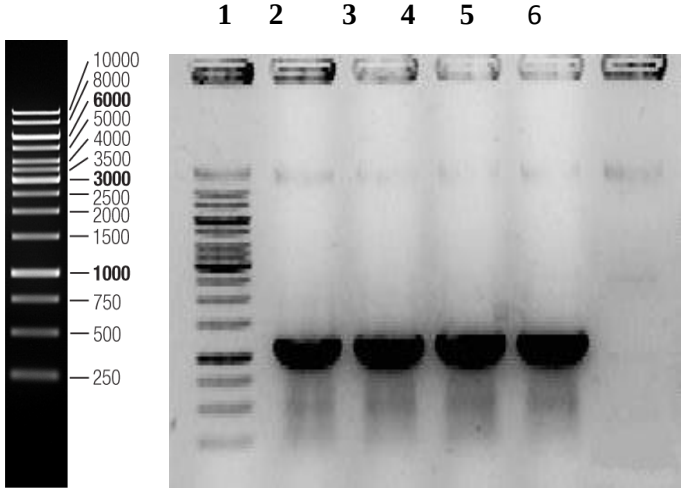

Figure 3: Colony PCR was performed using PPInk1200 primers and confirmation of the presence of recombinant pCAMB-hmPInk plasmid in E.coli strain JM109 (1: 10kb DNA ladder, 2, 3, 4 and 5 and 5: Colony PCR pCAMB-hmPInk plasmid in a number of transgenic E. coli strains JM109). And 6: negative control.
(1% agarose, V100 voltage, TAE 1X buffer and GeneRuler 1 kb DNA Ladder, ready-to-use (Thermo Scientific™)

For further confirmation, the extracted recombinant plasmid pCAMB-hmPInk in the positive clones was enzymatically specific digested using KpnI and XbaI restriction enzymes, which confirmed the enzymatic digestion and isolation of the cloned 968bp fragment in the pBGgHg vector (Fig. 4).

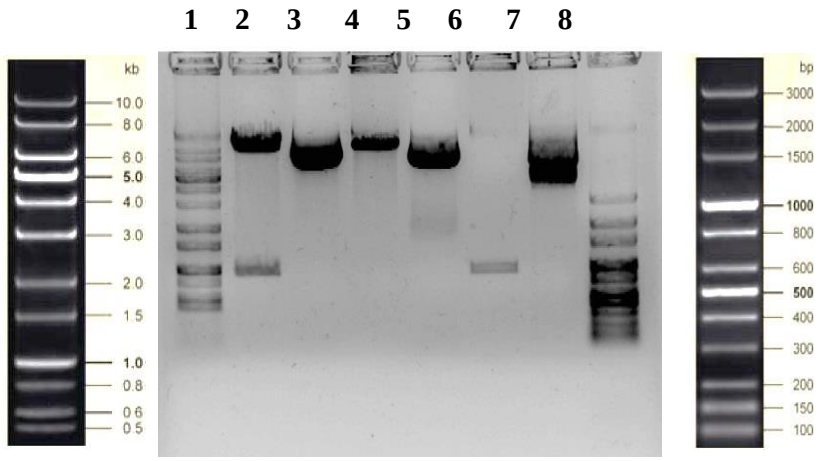

Figure 4: Enzymatic Double Digestion of Recombinant Plasmid pCAMB-hmPInk with Two restriction Enzymes KpnI and XbaI.(1: 10kb DNA ladder, 2: double digestion of pCAMB-hmPInk plasmid with two KpnI and XbaI enzymes, 3: pCAMB-hmPInk plasmid, 4: large linear fragment of pBGghg plasmid resulting from double digestion with two KpnI and X enzymes : Plasmid pBGghg, 6: digested fragment containing promoter and mini-proinsulin gene from plasmid TAV2 + (PPInk) by two KpnI and XbaI restriction enzymes, 7: plasmid TAV2 + (PPInk), 8: 3kb DNA ladder) (1% agarose, V100 voltage, TAE 1X buffer and Mid-Range DNA Ladder 3kb and 10kb (Jena Bioscience GmbH)

Conclusion

Higher mushroom like *P. eryngii* is a valuable bioreactor to produce therapeutic proteins; it is well known only for its natural forms in potential medical function. In addition to its original medicinal application and advantage of no side effects, transgenic *P. eryngii* is similarly a new approach of oral insulin. Our experimental strategy was to use *P. eryngii* to deliver both the exogenous mini-proinsulin and other natural medicinal contents of this mushroom to treat diabetes. Under the new concept of insulin coating, mini-proinsulin was not only produced within the cells, but also conserved by the natural structure of the cell wall, plasma membrane, and endoplasmic rectum. Thus, expressing mini-proinsulin in the bioreactor *P. eryngii* provides a novel strategy in oral administration of insulin. In contrast to the CTB-INS fusion protein expressed in transgenic potato plants [18], transgenic *P. eryngii* may also reduce blood glucose These experimental results provide a new direction for the treatment of diabetes.

REFERENCES

1. Abolliel, A. A., & Zedan, H. (2015). Synthesis, cloning and expression of a novel pre-miniproinsulin analogue gene in Escherichia coli. *Journal of advanced research, 6*(5), 663-671.
2. Cunha, N., Araújo, A., Leite, A., Murad, A., Vianna, G., & Rech, E. (2010). Correct targeting of proinsulin in protein storage vacuoles of transgenic soybean seeds. *Genet Mol Res, 9*(2), 1163-1170.
3. De Groot, M. J., Bundock, P., Hooykaas, P. J., & Beijersbergen, A. G. (1998). Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nature biotechnology, 16*(9), 839-842.
4. Ohga, S., & Kitamoto, Y. (1997). XVI. Future of mushroom production and biotechnology. *Food Reviews International, 13*(3), 461-469.
5. FAOSTAT. (2015). Food and Agriculture Organization of the United Nations, FAOSTAT database.
6. Sharma, K. K., & Kuhad, R. C. (2010). Genetic transformation of lignin degrading fungi facilitated by *Agrobacterium tumefaciens*. *BMC biotechnology, 10*(1), 67.
7. Chen, X., Stone, M., Schlagnhaufer, C., & Romaine, C. P. (2000). A fruiting body tissue method for efficient Agrobacterium-mediated transformation of *Agaricus bisporus*. *Appl. Environ. Microbiol., 66*(10), 4510-4513.
8. Molloy, S. (2004). Sugar transport and water relations of Agaricus bisporus.
9. Horgen, P. A., Kokurewicz, K. F., & Anderson, J. B. (1989). The germination of basidiospores from commercial and wild-collected isolates of *Agaricus bisporus* (= A. brunnescens). *Canadian journal of microbiology, 35*(4), 492-498.
10. Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M., & Porro, D. (2012). Recombinant protein production in yeasts. In *Recombinant gene expression* (pp. 329-358): Springer.
11. Kim, S., Ha, B.-S., & Ro, H.-S. (2015). Current technologies and related issues for mushroom transformation. *Mycobiology, 43*(1), 1-8.
12. Ni, T., Hu, Y., Sun, L., Chen, X., Zhong, J., Ma, H., & Lin, Z. (2007). Oral route of mini-proinsulin-expressing *Ganoderma lucidum* decreases blood glucose level in streptozocin-induced diabetic rats. *International journal of molecular medicine, 20*(1), 45-51.
13. Chang, S.-G., KIM, D.-Y., CHOI, K.-D., SHIN, J.-M., & SHIN, H.-C. (1998). Human insulin production from a novel mini-proinsulin which has high receptor-binding activity. *Biochemical Journal, 329*(3), 631-635.
14. Bartels, A.-K., Gottert, S., Desel, C., Schäfer, M., Krossa, S., Scheidig, A. J., . . . Lorenzen, I. (2019). KDEL Receptor 1 Contributes to Cell Surface Association of Protein Disulfide Isomerases.

15. Hefti, M. H., Dixon, R., & Vervoort, J. (2001). A novel purification method for histidine-tagged proteins containing a thrombin cleavage site. *Analytical biochemistry, 295*(2), 180-185.
16. Hinz, M., Gottschling, D., Eritja, R., & Seliger, H. (2000). Synthesis and properties of 2′-deoxycytidine triphosphate carrying c-myc tag sequence. *Nucleosides, Nucleotides and Nucleic Acids, 19*(10-12), 1543-1552.
17. Hua, Q.-x., Nakagawa, S. H., Jia, W., Huang, K., Phillips, N. B., Hu, S.-q., & Weiss, M. A. (2008). Design of an Active Ultrastable Single-chain Insulin Analog synthesis, structure, and therapeutic implications. *Journal of Biological Chemistry, 283*(21), 14703-14716.
18. Arakawa, T., Yu, J., Chong, D. K., Hough, J., Engen, P. C., & Langridge, W. H. (1998). A plant-based cholera toxin B subunit–insulin fusion protein protects against the development of autoimmune diabetes. *Nature biotechnology, 16*(10), 934-938.