Cloning and Expression of HBcAg Using Food Grade Vector pNZ8149 in *Lactococcus Lactis* for the Development of HBV Therapeutic Vaccine

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Abstract. Hepatitis B virus (HBV) infection remains a major cause of chronic liver diseases which may develop into cirrhosis and hepatocellular carcinoma (HCC). Although HBV prophylactic vaccine is available, currently there is no complete cure for chronic hepatitis B (CHB). One of the alternatives for CHB treatment is therapeutic vaccine. The HBV core antigen (HBcAg) becomes a suitable candidate for HBV therapeutic vaccine since it is known as a potent inducer of B-cell and T-cell responses. In this study, we report the development of HBV therapeutic vaccine by expressing HBcAg derived from HBV subgenotype B3, which is one of the predominant sub-genotypes in Indonesia. A food grade expression vector pNZ8149 which contains nisin-controlled gene as an inducible promoter, was used to express HBcAg in *Lactococcus lactis* NZ3900. Moreover, signal peptide usp45 was added in the N-terminal of the gene of interest to increase protein secretion efficiency. The native HBV core gene was synthesized and successfully cloned into plasmid pNZ8149 and transformed into *Lactococcus lactis* NZ3900 using lactose as a selection marker. The expression of HBcAg was induced using 10 ng/mL nisin and confirmed using dot blot hybridization analysis. Finally, the target protein which is 21 kDa in size was obtained by partial purification using size exclusion chromatography. In conclusion, HBcAg was successfully cloned and expressed using a food grade expression vector pNZ8149 in *Lactococcus lactis* NZ3900. These findings will pave the development of therapeutic vaccine for CHB treatment in the future.

Keywords: HBV · CHB · HBcAg · pNZ8149 · usp45 · *Lactococcus lactis* NZ3900 · nisin

1 Introduction

Hepatitis B virus (HBV) infection occurs as an acute hepatitis B (AHB) or chronic hepatitis B (CHB). Commonly, AHB recovers spontaneously after treatments, but on

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the other side CHB is one of the major causes of chronic liver diseases and may develop into cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. According to the World Health Organization (WHO), about 296 million people worldwide have been infected with HBV in 2019, and caused approximately 820.000 deaths, mostly from complications including cirrhosis and HCC. In addition, around 1.5 million new HBV infections have been reported each year [3]. HBV belongs to *Hepadnaviridae* family with around 3,200 bp of circular and partially double-stranded DNA genome. Nine HBV genotypes (A-I) and a new proposed genotype J have been classified based on > 7.5% nucleotide differences of HBV genomic sequences. Some of these genotypes are further characterized as sub-genotypes [4].

HBV replication involves reverse transcription of pre genomic RNA (pgRNA) intermediate into HBV DNA [5]. The covalently closed circular DNA (cccDNA) acts as an HBV transcriptional template and persistently exists in hepatocyte nucleus as a chromosome-like structure. HBV consists of four overlapping open reading frames (ORFs) including C, S, P and X which produce seven proteins. The C ORF encodes hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg). The S ORF encodes three viral envelope proteins including large, medium, and small hepatitis B surface antigen (HBsAg). The P ORF produces viral polymerase. The X ORF is responsible for expressing HBx protein which has multiple functions including signal transduction and transcriptional activation [6].

Currently, HBV prophylactic vaccine is available as a prevention for HBV infection, and drugs such as nucleos(t)ide analogues (NAs) and interferon alpha (IFN-α) are authorized as treatments for CHB [7]. However, a long-term therapy is needed to suppress HBV viral replication. Moreover, HBV viral load rebound often occur during therapeutic cessation due to the presence of cccDNA in hepatocytes. Therefore, new therapeutic approach is needed for CHB treatment [8]. Host immune response plays important roles for viral clearance in AHB. On the contrary, CHB is characterized with immune tolerance and exhaustion of HBV specific T-cells. Thus, overcoming immune tolerance during CHB has become the fundament for the development of HBV therapeutic vaccine [9], which aims to endeavor the enhancement of immune response by providing non-infective HBV antigen during CHB infection [10].

HBV core protein forms the inner core of HBV viral particles and produces HBcAg which is highly immunogenic due to its polymeric nature and the presence of a large number of T-cell epitopes [11]. It has been reported that HBcAg can induce strong immune responses in both T-cell dependent and T-cell independent manner which indicates that HBcAg acts as a potent B-cells activator [12]. Moreover, HBcAg is well presented by antigen presenting cells (APCs) [13]. Indeed, HBcAg specific immune response is essential for controlling HBV replication and preventing liver diseases [14]. HBcAg is a polypeptide with approximately 21 kDa in size which consists of 183 – 185 amino acids residues and can self-assemble into 27 nm particles [15–17]. The expressions of HBcAg recombinant protein in bacteria [18–20] and yeast [21–23] have been reported in various studies. In this study, we report the expression of HBcAg protein derived from HBV sub-genotype B3, one of the predominant sub-genotypes in Indonesia [24–28], in *Lactococcus lactis*.

Lactococcus lactis is a member of non-pathogenic gram-positive lactic acid bacteria (LAB) which initially used in dairy product fermentation. *Lactococcus lactis* is generally recognized as safe (GRAS) status, therefore, it is considered as one of the potential cell factories for the expression and production of recombinant therapeutic proteins. Other advantages of using *Lactococcus lactis* as a promising host expression system are the absence of endotoxins, and the availability of various cloning and inducible expression systems [29].

An example of successful expression system using *Lactococcus lactis* is the nisin controlled gene expression (NICE) system. Nisin is an anti-microbial peptide (lantibiotic) harboring two promoters: PnisA and PnisF. Since nisin can perform auto induction mechanisms, the gene of interest can be placed downstream of PnisA promoter within a plasmid and the gene expression can be induced by a certain amount of nisin [30]. The NICE plasmids and compatible expression host strains are developed and established by NIZO food research (Netherlands) [31]. Here, we used plasmid pNZ8149, which is considered as food grade safe since it retains LacF gene which can be selected by the addition of lactose instead of using antibiotic resistance gene [32]. However, consequently plasmid pNZ8149 needs a host strain which has lactose operon but without LacF gene. These characteristics can be found in *Lactococcus lactis* NZ3900 derived from a plasmid free progeny *Lactococcus lactis* subsp. *Cremoris* MG1363 [33].

Currently, only limited proteins have been known to be secreted by *Lactococcus lactis*, which will give advantage during the purification of recombinant protein. The signal peptide usp45 encodes the major extracellular protein in *Lactococcus lactis* and has been extensively used to lead the secretion of target protein in *Lactococcus lactis* [34]. Since we expect that HBcAg protein will be secreted extracellularly in the culture medium, signal peptide usp45 was added in the N-terminal of HBcAg gene to give a better secretion efficiency [35]. All in all, in this study we aim to produce HBcAg by exploiting the characteristics of *Lactococcus lactis* as a GRAS expression system, for the development of HBV therapeutic vaccine.

2 Materials and Methods

2.1 Plasmids, Bacterial Strains, and Culture Condition

Plasmids and bacterial strains used in this study are listed in Table 1. The pMAT plasmid was used as a cloning vector. The pNZ8149 plasmid was used as an expression vector. *Escherichia coli* TOP10 was propagated in Luria Bertani (LB) medium in the 37 °C shaker incubator with 200 rpm agitation. *Lactococcus lactis* NZ3900 were cultivated in M17 medium at 30 °C without agitation. Elliker agar medium was used for transformant screening. Ampicillin (100 μg/mL) and lactose (0.5%) were used as selection markers during cloning, while nisin was used as an inducer during protein expression.

Table 1. Plasmids and bacterial strains used in this study.

	Characteristics	Source
Plasmids		
pMAT	Ampicillin resistance	Invitrogen
pNZ8149	Lactose selection marker, nisin inducible promoter	MoBiTec GmbH
Bacterial strains		
Escherichia coli TOP10	Cloning host strain	Novagen
Lactococcus lactis NZ3900	Expression host strain	MoBiTec GmbH

2.2 Synthetic Gene

The native HBcAg gene (549 bp) was synthesized based on the sequence of HBV core region isolate 1839 Java (GeneBank EF473972.1) [36]. The signal peptide (SP) usp45 gene (81 bp) was synthesized according to the sequence from GenBank M60178.1 [34] and introduced into the N-terminal of HBcAg gene. The synthetic genes were produced and fused by Integrated DNA Technology Inc (USA) and packed into pMAT plasmid.

2.3 Cloning of PNZ8149-Usp45-HBcAg

The pMAT-usp45-HBcAg plasmid was first cloned into *Escherichia coli* TOP10 by heat shock method [37] and the positive clones were selected using 100 μg/mL ampicillin. Next, pMAT-usp45-HBcAg plasmid was isolated using Plasmid MIDI kit (Qiagen) and restricted using NcoI and XbaI endonuclease enzymes. The fragments were purified using DNA purification kit (Qiagen) and ligated into pNZ8149 plasmid which already linearized using the same endonuclease enzymes. The ligated fragments were further purified using DNA purification kit (Qiagen) and directly transformed into *Lactococcus lactis* NZ3900.

2.4 Transformation of PNZ8149-Usp45-HBcAg into Lactococcus Lactis NZ3900

Lactococcus lactis NZ3900 cells were grown by sequential inoculation of bacterial stock into 5 mL, 25 mL, and 100 mL of M17 medium containing 2.5% (w/v) glycine, 0.5 M sucrose and 0.5% (w/v) glucose until optical density at 600 nm (OD600) ~ 0.3 was reached. The *Lactococcus lactis* NZ3900 competent cells were prepared by harvesting the cells using centrifugation (6,000 × *g*) at 4 °C for 10 min to obtain pellet, followed by resuspension using buffer A (0.5 M sucrose and 10% (v/v) glycerol). The resuspended pellet was re-centrifuged (6,000 × *g*) at 4 °C for 10 min followed by resuspension using buffer B (0.5 M sucrose, 10% (v/v) glycerol, and 0.05 M EDTA) and incubated on ice for 15 min. The suspension was re-centrifuged (6,000 × *g*) at 4 °C for 10 min followed by first dilution using 15 mL of buffer A. Finally, the diluted cells were re-centrifuged (6,000 × *g*) at 4 °C for 10 min followed by second dilution using 1 mL of buffer A. The ligated DNA (pNZ8149-usp45-HBcAg) was added into 100 μL of competent cells

and subjected into electroporation method (2000 V, 25 μF, 200 Ω). The transformed cells were immediately transferred into 400 μL of M17 medium containing 0.5% (w/v) glucose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$ and incubated on ice for 5 min followed by 3 h incubation at 30 °C without agitation.

For screening, the cells were plated onto Elliker agar medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.4% (w/v) sodium chloride, 0.15% (w/v) sodium acetate, 0.05% (w/v) L-ascorbic acid, 1.5% (w/v) agar, 0.5% (w/v) lactose and 0,004% (w/v) bromocresol purple) and incubated for 1 – 2 days at 30 °C without agitation [38]. The transformant cells were screened by PCR using forward primer PnisA-F (5'-TTC CCT CGA GGG ATC TAG TCT TAT AAC-3') and reverse primer TpNZ8149-R (5'-CGA AAG CGA AAT CAA ACG-3'), with the following PCR conditions: pre-denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 30 s and post elongation at 72 °C for 6 min.

The positive colonies were inoculated into M17 medium supplemented with 0.5% (w/v) lactose. The cells were harvested, and the pellet was initially washed using STE buffer (6.7% (w/v) saccharose, 50 mM Tris-HCl pH 8.0, 1 mM EDTA) [39] and followed by the recombinant plasmids isolation using Qiagen Plasmid MIDI kit (Qiagen) according to the manufacture's protocol with the addition of 10 mg/mL of lysozyme into lysis buffer. Further confirmations were done by plasmid restriction using nuclease enzymes mentioned above and sequencing.

2.5 Inducible Expression of HBcAg in Lactococcus Lactis NZ3900

Protein expression was performed according to the previous method [35] with some modifications. Firstly, *Lactococcus lactis* NZ3900 cells harboring recombinant pNZ8149-usp45-HBcAg were cultivated in M17 media supplemented with 0.5% lactose at 30 °C without agitation for 2–3 h until OD600 ~ 0.4 – 0.5 was reached. A various concentration of nisin (5 ng/mL, 10 ng/mL, and 50 ng/mL) were added for induction. The cells were harvested, and the supernatant was collected 5 h later by centrifugation at 12,000 × *g*, 4 °C for 30 min, followed by protein precipitation using 45% ammonium sulfate according to the method mentioned elsewhere [40] for overnight at 4 °C. The precipitated protein was collected by centrifugation at 12,000 × *g*, 4 °C for 30 min and the obtained pellet was resuspended with 50 mM Tris-HCl pH 7.8.

The total protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific) as described in the manual's instructions. Furthermore, dot blot hybridization assay was also carried out according to previous protocol [37]. Samples were dropped onto nitrocellulose membrane, then blocked with 10% skim milk for one hour followed by incubation with mouse anti-HBcAg antibody (1:1000) as primary antibody. The membrane was then rinsed with TBE-0.1% Tween, followed by the incubation with anti-mouse IgG as secondary antibody and the signals were developed using BCIP-NBT (5-bromo-4-chloro-3-indoly/phosphate/nitro blue tetrazolium) substrate (Sigma).

2.6 Partial Purification Using Size Exclusion Chromatography

Protein purification was performed using size exclusion chromatography according to the previous method [41]. Samples were loaded onto Sephadex G-50 pre-packed column which had been pre-equilibrated with 50 mM Tris-HCl pH 7.8 using a flow rate of 0.1 mL/min at 4 °C. Fractions were collected and analyzed by spectrophotometer at absorbance 280 nm. To confirm the protein expression, the fractions were subjected into SDS-PAGE using 12% polyacrylamide gel and stained by silver staining kit (Thermofisher Scientific). Dot blot analysis was also performed for the selected fractions.

3 Results

3.1 Cloning of PNZ8149-Usp45-HBcAg

The synthetic HBcAg gene was fused with usp45 at its N-terminal, packed into pMAT plasmid, and propagated into *Escherichia coli* TOP 10. The isolated pMAT-usp45-HBcAg plasmid was restricted using NcoI and XbaI endonuclease enzymes and inserted into the MCS of pNZ8149 expression vector to generate pNZ8149-usp45-HBcAg. The map of pNZ8149-usp45-HBcAg construct is shown in Fig. 1.

3.2 Transformation of PNZ8149-Usp45-HBcAg into Lactococcus Lactis NZ3900

The pNZ8149-usp45-HBcAg construct was transformed into *Lactococcus lactis* NZ3900 and the positive transformants which produced yellow color colonies were selected using 0.5% lactose in the Elliker agar medium (Fig. 2a) and confirmed using PCR (Fig. 2b). The recombinant plasmid was isolated and the inserted HBcAg gene was confirmed by enzyme digestion using NcoI and XbaI endonuclease enzymes. The size of the construct was 3815 bp with 630 bp of inserted gene (Fig. 2c). The orientation of HBcAg nucleotide sequence was also confirmed by sequencing analysis and no mutation was detected within the sequence. HBcAg has an open reading frame (ORF) of 549 bp that encodes 183 amino acid residues with a predicted molecular weight 21 kDa. Translation analysis showed that there was no amino acid substitution, which indicated that the inserted HBcAg gene encoded amino acids with the exact similarity with the

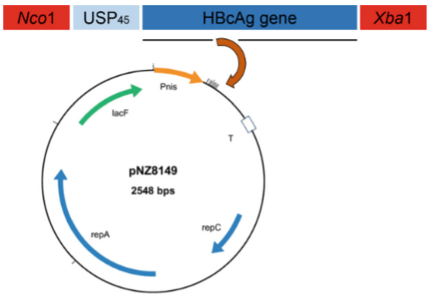

Fig. 1. The map of pNZ8149-usp45-HBcAg construct. The native HBcAg gene along with SP usp45 gene were inserted into plasmid pNZ8149.

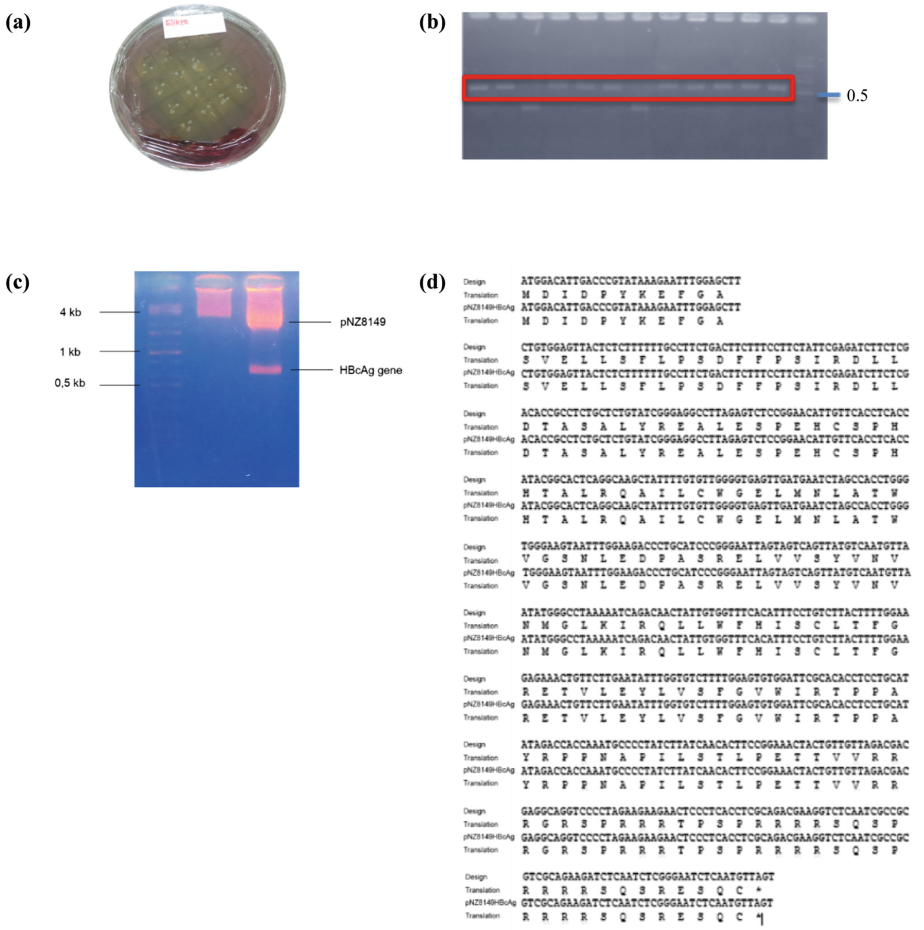

Fig. 2. Transformation of pNZ8149-usp45-HBcAg into *Lactococcus lactis* NZ3900. (a). Positive transformants harboring pNZ8149-usp45-HBcAg were indicated as colonies which showed yellow color in Elliker agar medium. **(b).** Screening of positive transformants using PCR method. **(c).** The plasmid pNZ8149-usp45-HBcAg was digested using NcoI and XbaI endonuclease enzymes. Lane 1: un-restricted, lane 2: restricted. The band (630 bp) confirmed that HBcAg gene was successfully inserted into food grade expression vector pNZ8149. **(d).** The comparison between HBcAg reference sequence and HBcAg within pNZ8149-usp45-HBcAg construct. Sequence analysis showed 100% similarity and no mutation had been detected both in nucleotide sequence and the translated amino acids.

reference sequence (Fig. 2d). These data suggest that the pNZ8149-usp45-HBcAg construct had been successfully transformed into *Lactococcus lactis* NZ3900 as expression host.

Table 2. The concentrations of total protein expressed in *Lactococcus lactis* NZ3900.

Nisin concentration (ng/mL)	Total protein (mg/mL)
Un-induced	1.3084
5	3.5324
10	4.1787
50	3.7307

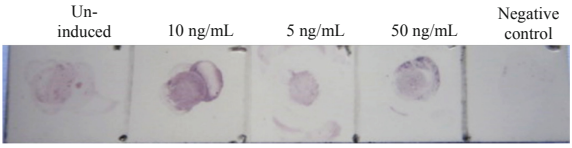

Fig. 3. Dot blot hybridization assay. The dot blot hybridization assay of HBcAg precipitated protein derived from samples induced by a various concentrations of nisin. Lane 1: un-induced; lane 2: 10 ng/mL; lane 3: 5 ng/mL; lane 4: 50 ng/mL; lane 5: negative control *Lactococcus lactis* pNZ8149.

3.3 Inducible Expression of HBcAg in Lactococcus Lactis NZ3900

The concentration of total protein from precipitated samples were measured using BCA kit (Table 2). Total protein was significantly increased in nisin-induced samples compared with un-induced control, even though the un-induced control still produced protein, possibly containing less HBcAg. The highest total protein (4.1787 mg/mL) was achieved with the induction of 10 ng/mL nisin.

To further confirm the expression of HBcAg following nisin induction at a various concentrations, the precipitated samples were subjected into dot blot hybridization analysis using HBcAg monoclonal antibody. The precipitated sample derived from *Lactococcus lactis* NZ3900 harboring only pNZ8149 was used as negative control. Signals were detected in un-induced, and all samples which were induced with nisin at concentration of 10 ng/mL, 5 ng/mL, and 50 ng/mL, respectively and there was no signal detected in negative control (Fig. 3).

3.4 Partial Purification of HBcAg Protein

The precipitated protein derived from sample induced with nisin (10 ng/mL) was subjected into size exclusion chromatography using 50 mM Tris-HCl pH 7.8 as eluent. The absorbance of the collected fractions was measured at 280 nm (Fig. 4a) and further analyzed using silver staining (Fig. 4b). Fractions 7 and 8 at 30 min and 35 min, respectively, showed target bands (21 kDa) and further confirmed using dot blot hybridization. As expected, dot blot analysis of fractions 7 and 8 showed clear signals, indicated the presence of HBcAg protein (Fig. 4c).

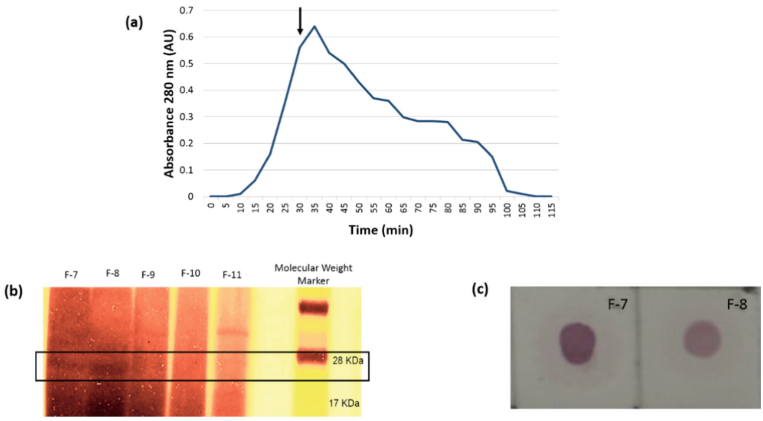

Fig. 4. Partial purification of HBcAg protein. The precipitated protein was subjected into size exclusion chromatography. **(a).** The absorbance of the collected fractions was measured by UV spectrophotometer at 280 nm. The arrow points to the fractions with the highest absorbance (Fraction 7 and 8 at 30 min and 35 min, respectively). **(b).** The obtained fractions (7–11) were subjected into silver staining. Fractions 7 and 8 showed the target bands at 21 kDa in size. **(c).** Fractions 7 and 8 were analyzed using dot blot hybridization and clear signals were detected in both fractions.

4 Discussion

In this study, we elaborate the potential of hepatitis virus core antigen (HBcAg) for the development of HBV therapeutic vaccine. Basically, the main goal of chronic hepatitis B (CHB) treatment is preventing the development of progressive liver diseases including cirrhosis and hepatocellular carcinoma. However, currently there is no complete cure available for CHB. Therefore, development of alternative therapy using new approach is urgently needed. It is well known that the humoral antibody response plays a role during HBV viral clearance and prevents the spreading of HBV infection within host. On the other side, the cellular immune response is responsible in the elimination of infected hepatocytes [42]. During acute hepatitis B (AHB) the HBV specific CD4 and CD8 T-cell responses are commonly observed, along with antiviral cytokines and specific antibodies against HBV. On the contrary, during CHB, the immune response is weak or often functionally impaired which is believed to be caused by a long-term exposure of HBV viral antigens [43]. Based on these, therapeutic vaccine has been proposed as a potential new treatment for CHB which aim to eradicate HBV persistent viral infection by stimulating immune responses.

Here, we selected HBcAg derived from HBV sub genotype B3 which prevalent in Indonesia [36] as the candidate for the development of HBV therapeutic vaccine. It has been reported that HBcAg can activate specific cytotoxic T lymphocyte (CTL) response and induces specific T-cells. In addition, HBcAg can activates B cells as antigen presentations cell (APC) and stimulates Toll-like receptor (TLR) [44, 45]. Therefore, HBcAg is qualified as potential candidate for HBV therapeutic vaccine aiming the stimulation of HBV specific T-cells immune response. Indeed, a new HBV therapeutic vaccine known as NASVAC which is generated based on the combination of hepatitis B virus

surface antigen (HBsAg) and HBcAg has been developed. The formulation of NASVAC has been administered intranasally during clinical trials phase I, II, and III. The data indicates that NASVAC is safe and successfully develop T-cells and B-cells responses. Moreover, NASVAC is also found to be immunogenic which is indicated by HBsAg and HBcAg seroconversions [46, 47].

Therefore, we focus on the optimization of HBcAg heterologous expression and secretion in *Lactococcus lactis* NZ3900. Conventionally, *Escherichia coli* is the most used expression host, however, it possesses some obstacles such as, the presence of endotoxin and lipopolysaccharides which should be removed prior to the administration in humans, thus can create a problem during downstream purification process. In contrast, the production of heterologous protein in *Lactococcus lactis* can be easily secreted into the medium, thus benefit the purification process [48]. *Lactococcus lactis* is a well characterized Gram-positive bacterium, which has relatively simple metabolism and its whole genome sequence is already well known. Since it has GRAS (generally recognized as safe) status, *Lactococcus lactis* is becoming an attractive host to produce antigenic and therapeutic proteins and peptides. Thus, it is also important to develop food grade vector system to lead the secretion of target protein [29].

To meet the safety requirement for a therapeutic product, food grade host expression system and consequently, food grade expression vector should be used. The design of protein recombinant construct should be intended to avoid the thread cause by antibiotic resistance selection markers which could impair therapeutic application [49]. Thus, expression vector should contain selection markers that are acceptable in food industry and described as food grade. Here, we use pNZ8149 vector which harbors lactose as complementation selection marker and has been considered as food grade selection marker. In this study, nisin controlled gene expression (NICE) expression system is used to produce HBcAg protein. The NICE system within pNZ8149 allows autoregulation of nisin biosynthesis in *Lactococcus lactis* NZ3900. Thus, protein expression can be achieved by assigning the target gene downstream of the nisin promoter followed by induction using a certain amount of nisin. Nisin, a small 34 amino acid bacteriocin, contains lanthionine residues and categorized as a lantibiotic which has a wide antimicrobial spectrum against Gram-positif bacteria [50, 51]. Nisin can be used to induce the expression of target gene using NisA promoter via membrane-associated histidine kinase sensor (NisK) and a transcriptional regulator (NisR). The signal transduction pathway starts from the binding of nisin to receptor NisK which lead to autophosphorylation. The phosphate group is transferred to NisR, followed by mRNA synthesis and ribosomal synthesis of target gene [30].

In this study, the HBcAg protein is secreted into the medium through signal peptide (SP) usp45 which is combined into nisin inducible pNZ8149 NICE system. The SP usp45 gene encodes the major extracellular protein produced by *Lactococcus lactis*, thus benefit the purification process [34]. Here, we describe the construction and production of HBcAg protein in NICE lactococcal food grade cloning and expression system based on the selection using lactose. The HBcAg gene is successfully cloned into pNZ8149 and expressed in *Lactococcus lactis* NZ3900. The highest total protein concentration is achieved by the addition of 10 ng/mL nisin. Moreover, partial purification with size

exclusion chromatography shows the target band (21 kDa in size) and the HBcAg protein shows reactogenicity with specific HBcAg antibody through dot blot hybridization analysis. These results indicate that the production of HBcAg in *Lactococcus lactis* using food grade expression vector could be further developed as the candidate of HBV therapeutic vaccine.

Since functional immune response is inevitable to successfully eradicate HBV infection, therapeutic vaccine is designed to enhance the efficacy of current HBV antiviral therapy and overcome the tolerogenic and exhaustion of immune responses. The ideal outcome should be the improvement of HBV specific immune responses in both innate and adaptive which lead to a strong specific T-cells and B-cells immunity against HBV antigens. However, it is worth noting that the induction of weak T-cells response should be avoided since instead of clearing the virus, it can cause liver tissue damage. Thus, it is important to develop HBV therapeutic vaccine which able to induce immune response without causing the progression of liver disease [52]. Further *in vivo* research is needed to evaluate the immunogenicity and characterize the function of HBcAg produced by the NICE lactococcal expression system. This will allow further development of the NICE lactococcal expression system as acceptable host to produce therapeutic proteins.

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