The Expression of Codon Optimised Hepatitis B Core Antigen (HBcAg) of Subgenotype B3 Open Reading Frame in *Lactococcus lactis*

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**Introduction**

Hepatitis B virus (HBV) is currently causing around million people worldwide with chronic HBV infection, while approximately another one million died each year on disease related to liver failure [1]. The HBV is classified into eight genotypes (A-H) based on nucleotide array and each genotype is classified into several subgenotypes. There are three dominant HBV genotypes in Indonesia, namely, HBV/B (70.9%), HBV/C (27.5%), and HBV/D (1.6%). HBV/B is the most dominant HBV genotype in Western Indonesia while HBV/B3 is the most HBV subgenotype in Java region [2, 3]. In other studies, analysis of 214 blood donors (age 18–64 years) and 171 patients with HBV-associated liver diseases samples from Makassar have been shown that HBV/B and HBV/C are dominant in which subgenotype B3 and C1 reach to 95% and 82.2%, respectively [4, 5]. The HBV is subjected as second carcinogen after tobacco, considering about 80% hepatocellular carcinoma related to HBV infection [6]. Milestone of chronic HBV treatments were adequately improved since 1990’s. Early on, most HBV's

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cases were treated using interferons and continued to the developments of pegylated interferon. However, their curative effect was far from expectation [7]. On the other hand, global Hepatitis A and B vaccination program contributes to global reduction of hepatitis infection. The CDC reported reduction of HBV incidents in relation to high HBV vaccination coverage.

As the member of Hepadnavirus family, Hepatitis B virion consists of outer lipid envelope and icosahedral nucleocapsid. The outer envelope (HBsAg) plays roles in binding and entry into susceptible cells, while the nucleocapsid (HBcAg) contains hepatitis B core antigen that simultaneously requires the presence of HBeAg to maintain replication. The interest on investigating HBcAg was began while HBcAg valued as the most immunogenic HBV element while HBV infection occurred, since it controls the work of dendritic cells (DCs) to efficiently presents antigens on the surface cells [8]. More deep investigation on DC’s function related HBV infection confirmed the failure of DC’s function in controlling HBV infection might causes liver pathology [9]. Thus, improving DC’s performance becomes most of target treatment in HBV immune therapy. However, there is a statement that both HBcAg and HBeAg cannot be recognized by DC. The inability of DC making response to the presence of HBcAg due to inappropriate uptakes of HBcAg that leads to failure in prompting DC’s antigen presentation. On the other hand, combination of HBcAg-HBsAg complexes efficiently can be presented by DC [10], thus a combo between HBsAg-HBcAg and interferon becomes our future target for HBV immune therapy. To reach the aim, every single antigen will be produced separately, and this work is the justification of production of single HBcAg antigen.

In this study, the HBcAg antigen is produced to meet the expectation that we might be able to improve the patients’ immune responses, more focus on repairing the dendritic cells duties in presenting the antigen on the cell surface. Hopefully, specific immune responses will be generated and has its consequences on improving patients’ survival rates. Earlier publications successfully expressing the HBV core protein in Escherichia coli [11], transgenic tobacco [12] and yeast Saccharomyces cerevisiae [13]. For this work, Lactococcus lactis was chosen as expression host in regard of safety concern and it is proven to have very high induction ratio (up to 10000 folds) [14].

Our previous work was also successful in expressing HBcAg in its native sequence using L. lactis as expression host. As the continuation of the work, we attempted to expand the expression by generating optimisation of the HBcAg codon, suitable for the L. lactis expression host to achieve higher rate protein expression. As it found that codon optimisation determine successful gene expression [15] by increasing translational efficiency [16]. To our knowledge, this is the first work of expressing HBcAg gene using L. lactis in the form of codon optimised.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were cultivated in Luria-Bertani (LB) medium at 37°C with aeration. L. lactis strains were cultivated in M17 Broth medium supplemented with 0.5% glucose at 30°C without aeration.

Design of Indonesian HBcAg synthetic gene and codon optimisation analysis using Geneious® software

The HBcAg gene used in this study is 549 bp-long synthetic gene of 1839 Java isolate of Hepatitis B virus

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains and plasmids used in the study.</th>
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<tbody>
<tr>
<td><strong>Strains and vector</strong></td>
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<tr>
<td><strong>Bacterial strains</strong></td>
</tr>
<tr>
<td>Escherichia coli MC 1061</td>
</tr>
<tr>
<td>Lactococcus lactis NZ3900</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
</tr>
<tr>
<td>pIDT</td>
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<tr>
<td>pHZ8148</td>
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Amp′: ampicillin resistance; Cmp′: chloramphenicol resistance

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(GenBank EF473972.1), while 81 bp-long signal peptide (SPusp45) (GenBank M60178.1) was added to the coding sequence. The codon optimisation of HbcAg gene was done by Integrated DNA Technologies Inc. (USA), the synthetic costumed made gene construction was arranged into : usp45—HbcAg sequence. Two restriction enzymes, NcoI and XbaI, were introduced at the -N and -C terminals, respectively. Delivered synthetic gene was attached into pIDT plasmid vector. Codon optimisation was then analysed using Geneious® software. Geneious® software allows us to align the optimised codon and using the native sequence as reference. Since it already accepted that codon usage is not random between species and compositional biases of the codon is the only one factor shaping the codon usage variations, which differ in an extreme AT or GC rich unicellular organism [17, 18].

Construction of pNZ8148-usp45-HBcAg and sub cloning into E. coli MC 1061

Prior to sub cloning into E. coli MC 1061, the pIDT plasmid carrying codon optimised HBcAg gene was cut with endonuclease enzymes NcoI and XbaI, to retrieve the HBcAg gene. Digested usp45—HBcAg (opt. codon) was then inserted into pNZ8148 plasmid vector, which already being linearized using NcoI and XbaI restriction enzymes. The shuttle vector with insert was then transformed into E. coli MC 1061 using Sambrook’s method [19]. Selected colonies were grown in Luria-Bertani (LB) agar media containing chloramphenicol, then analysed using PCR amplification. Primer used in this study was PnisA_F (5'-TTC CCT CGA GGG ATC TAG TCT TAT AAC-3’) and TpNZ8148_R (5’-GCT AAA ACG TCT CAG AAA CG-3’) primers. PCR amplification was done as follows: pre-denaturation at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 30 sec, followed by a final elongation at 72°C for 6 min.

Transformation of pNZ8148-usp45-HBcAg into Lactococcus lactis NZ3900

The recombinant pNZ8148 plasmid anchoring the usp45-HBcAg (opt. codon) gene was inserted into L. lactis NZ3900 by means of electroporation, then was grown overnight in M17 media (0.5% glucose, 0.5 M sucrose, 25% Glycine). As much as 10% of overnight culture was inoculated into fresh media to be grown at 30°C until it reach an OD₆₀₀ = 0.3. The culture was processed by centrifugation at 6000 ×g for 10 min to obtain pellet. Once centrifugation was done, pellet was resuspended with washing buffer (0.5 M sucrose, 10% glycerol). Resuspended-pellet was centrifuged for second times, before it was diluted using buffer containing 0.5 M sucrose, 10% glycerol and 10 mM EDTA, followed by incubation on ice for 15 min. Samples were processed further by centrifugation at 6000 ×g, 10 min before it was resuspended with 1 ml of wash buffer. DNA was inserted into L. lactis NZ3900 by through electroporation at conditions 2000 V, 25 µF, 200 Ω. Transformants selection was using M17 media containing 0.5% glucose and 100 µg/ml chloramphenicol. The selection plates were incubated at 30°C for 16 h, with observation for 24 until 48 h, then analysed using NcoI-XbaI enzymes pair, to identify colonies holding targeted insert. The sequence of the construct was verified by sequencing.

Protein production and purification

Inoculation of 10% overnight culture of L. lactis NZ3900-pNZ8148-usp45-HbcAg (native and opt. codon) into M17 media (0.5% glucose) was set at 30°C for 2 h until the culture reach an OD₆₀₀ at 0.5. Nisin induction was done at final concentration of 5, 10, and 50 ng/ml. Uninduced culture was prepared as control. Protein of interest was collected with centrifugation at 12,000 ×g, 4°C for 30 min, through the centrifugation, supernatant was able to be collected. Supernatant from native and codon optimised HBcAg were tested its total protein by BCA assay. HBcAg presence was also identified by slot blot hybridization. Then, supernatant brought to 45% saturation by adding ammonium sulphate and overnight incubation at 4°C. Precipitated protein was collected by centrifugation at 12,000 ×g, 4°C for 30 min, then resuspended with Tris HCl 50 mM pH 7.4. Resuspended sample was injected to gel filtration chromatography process using Sephadex G-50. Sephadex resin was equilibrated with Tris HCl 50 mM pH 7.4. Protein flow rate was 1 ml/min at 4°C. Purified sample was analysed with spectrophotometer at absorbance 280 nm.

BCA assay

Protein concentration determination was carried out using BCA protein assay kit (Pierce Biotechnology,
2013) with bovine serum albumin (BSA) as protein standard. Serial BSA dilution at 2000 µg/ml, 1500 µg/ml, 1000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 25 µg/ml was made to build linear regression. Protein sample was mixed with working reaction into 96-well plate (1:20), then incubated at 37°C for 30 min. Quantification by ELISA reader was done at $\lambda = 540$ nm [20].

### Protein detection and characterization using Western Blot, Slot Blot, Dot blot hybridizations, and silver staining method

Western Blot, Slot Blot, and Dot Blot hybridization assays were done to confirm the presence of protein target. Slot blot method was applied according to Mannoles and Bartelt (1996) with some modification. The suction was turned on and blot was removed from the manifold. Purified protein was separated using 12% SDS-PAGE then followed by Western Blot analysis, following protein blotting guide (published by BioRad). Separated protein on acrylamide gel was transferred into nitrocellulose membrane for western technique, on the other hand the purified protein directly applied onto nitrocellulose membrane for dot blot analysis. All nitrocellulose membranes were then incubated with 10% skim milk for an hour prior to primary antibody incubation. The mouse anti-HBcAg antibody (GE Healthcare, UK) was used as primary antibody (diluted in 1:1000). After primary incubation, the membrane washed with TBS-T, 3 times 5 min each. Secondary antibody incubation for an hour using Anti mouse IgG-AP conjugate (1:1000). The signal was developed using BCIP/NBT (5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium) substrate (SigmaFast). The protein staining with silver stain was following manufacturer’s instruction as written in Pierce™ Silver Stain kit by Thermo Fisher company.

![Fig. 1. Alignment between HBcAg native and HBcAg codon optimised.](http://dx.doi.org/10.4014/mbl.1806.06004)
Results

Codon optimisation analysis using Geneious® software

The alignment depicts changes for approximately 25% bases (shown by red circle on Fig. 1) to make the sequence more susceptible to be expressed in Lactococcus lactis (Fig. 1). The L. lactis is AT rich organism, normally it has GC content around 35% [21]. In this research we were introducing insert sequence that has higher GC content (47.8%), as shown in Fig. 1.

Synthetic gene and construction of usp45-HBcAg into pNZ8148 expression vector

The synthetic core gene of hepatitis virus (HBcAg) was fused with peptide signal usp45 that is commonly arranged at the -N terminal site. The usp45 signal peptide will deliver the protein to the place where protein precursor available, which is in cytoplasmic membrane [19]. In this research, the usp45 gene was kept in native sequence, while the HBcAg gene was optimised with the suggestion of the synthetic gene provider. The optimisation of the codon usage is in the request for the most suitable form to be expressed in L. lactis expression host.

Delivered synthetic gene was attached into pIDT plasmid vector. The pIDT plasmid vector was separated with HBcAg gene. Electrophoresis confirms the presence of ±2700 bp vector (pIDT) band with ±750 bp HBcAg inserted gene which being optimised. The gene was lined in the sequence of NcoI-usp45-HBcAg-XbaI then ligated into pNZ8148 plasmid (Fig. 2).

The pNZ8148-usp45-HBcAg transformation into Escherichia coli MC 1061

Once the restriction product of usp45-HBcAg from pIDT vector was already inserted into pNZ8148 plasmid, the transformation into E. coli MC 1061 was done using heat shock method. Clone selection of E. coli MC 1061 that carrying recombinant plasmid pNZ8148 was done using plate selection whereas positive clone will have white colour (data not shown). The white-coloured clones were analysed with PCR to confirm the HBcAg gene attached in the plasmid pNZ8148.

Transformation of pNZ8148-usp45-HBcAg into Lactococcus lactis NZ3900

Once the HBcAg in pNZ8148 was established in E. coli MC 1061, it was followed by transformation into L. lactis NZ3900. The NZ3900 has specific features where the LacF gene is deleted and integrated into chromosome, this makes them unable takes up lactose even it is available in the media [22]. Selection of transformation products was done with M17+0.5% glucose media that contains 100 µg/ml chloramphenicol. Selected colonies were then isolated and being cut using NcoI and XbaI (as seen on Fig. 3). Sequencing of the selected clones comes out with the result that there is no mutation occurred, as well as confirmation of homology status to HBcAg native type adr4 (Fig. 4).
Protein expression, purification and characterization

The protein expression in lactic acid bacteria (LAB) is tightly regulated, which can hold toxic and essential proteins. It is easy to be expanded in a large-scale expression and the expression process itself is quite simple. In our research, the expression was induced by 10 ng/ml Fig. 4. BLAST tool confirms sequencing result identical for 100% to hepatitis core capsid protein.

![BLAST result](image)

Fig. 4. BLAST tool confirms sequencing result identical for 100% to hepatitis core capsid protein.

![Graphical representation](image)

Fig. 5. (A) Chart column comparison between native and codon optimised HBcAg gene in *L. lactis* bacteria. Both groups were treated with the same concentration of Nisin inducer. The group which having optimised gene constantly showing higher expression of protein, except for non-induction group which was only shows small gap between native and codon optimised HBcAg groups. The values of total protein for each treatment group is the mean of three times replications. (B) Protein characterization using Slot Blot method. 1, 2, and 3 = native with 5, 10 and 50 ng/ml of inducer; 4, 5, and 6 = codon optimised with 5, 10 and 50 ng/ml of inducer.

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The total protein was depicted on column graph below (Fig. 5A). Calculation of total protein shows that overall protein expression was improved, expression of native HBcAg gene reached 8.250 mg/ml (for total protein), on the other hand the optimised HBcAg gene expression elevated 9.271 mg/ml.

Slot blot result shows that all conditions have positive results which indicate that HBcAg protein is produced (Fig. 5B). Quantification reveals that the optimised codon has higher expression level comparing to the native. The increment level is determined by dividing AUC value of codon optimised with that of native. Highest expression level of codon optimised is obtained at 50 ng/ml of inducer (Table 2).

Prior to purification, sample was treated using ammonium sulftate precipitation. During purification, 10 fractions were collected from sephadex G-50. Absorbance of each fraction were checked (Fig. 6). Then all fractions were run in SDS-PAGE with 12% separating gel to optimise the protein separation. Detection of fractionated

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**Table 2. Native and codon optimised HBcAg expression level determination.**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Inducer (ng/ml)</th>
<th>Area Under Curve (AUC)</th>
<th>AUC Means</th>
<th>Increment level&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>5.0</td>
<td>13,333,350.0</td>
<td>12,727,572.0</td>
<td>12,663,693.0</td>
</tr>
<tr>
<td>Native</td>
<td>10.0</td>
<td>14,835,593.0</td>
<td>13,861,765.0</td>
<td>13,404,522.0</td>
</tr>
<tr>
<td>Native</td>
<td>50.0</td>
<td>15,261,664.0</td>
<td>14,147,543.0</td>
<td>14,296,371.0</td>
</tr>
<tr>
<td>Optimised</td>
<td>5.0</td>
<td>25,245,922.0</td>
<td>25,362,337.0</td>
<td>17,259,274.0</td>
</tr>
<tr>
<td>Optimised</td>
<td>10.0</td>
<td>22,792,508.0</td>
<td>23,075,659.0</td>
<td>18,197,174.0</td>
</tr>
<tr>
<td>Optimised</td>
<td>50.0</td>
<td>19,369,659.0</td>
<td>101,858,448.0</td>
<td>16,546,697.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Comparing to native in the same condition

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**Fig. 6. Absorbance value and total protein concentration of ten fractions from filtration gel chromatography.**

final concentrations of Nisin inducer. The total protein was depicted on column graph below (Fig. 5A). Calculation of total protein shows that overall protein expression was improved, expression of native HBcAg gene reached 8.250 mg/ml (for total protein), on the other hand the optimised HBcAg gene expression elevated 9.271 mg/ml.

Slot blot result shows that all conditions have positive results which indicate that HBcAg protein is produced (Fig. 5B). Quantification reveals that the optimised codon has higher expression level comparing to the native. The increment level is determined by dividing AUC value of codon optimised with that of native. Highest expression level of codon optimised is obtained at 50 ng/ml of inducer (Table 2).

Prior to purification, sample was treated using ammonium sulftate precipitation. During purification, 10 fractions were collected from sephadex G-50. Absorbance of each fraction were checked (Fig. 6). Then all fractions were run in SDS-PAGE with 12% separating gel to optimise the protein separation. Detection of fractionated

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**Fig. 7. (A) First line of protein characterization of all collected fractions from sephadex G-50 were tested using silver staining.**

As seen on the picture, fraction 3, 4, 5 closely depicts protein bands around the size of expected protein. (B) Dot blot hybridization, (C) Western blotting techniques. The dot blot hybridization using anti-HBcAg confirms almost all of fractions were showing positive results, however, more specific western blotting, whereas protein gone through separation based on their molecular weight, confirms that there were only fractions 4 and 5 have positive bands at the desired molecular weight.
samples was done using silver staining. Results clearly show protein bands at approximately 21 kDa from fraction 3, 4, and 5. The other fractions (fractions 6 to 10) were showing protein bands which are not the protein of interest due to its size higher than 21 kDa. Further characterization of protein of interests was done using western blotting and dot blot hybridizations. The anti-HBcAg recognised the protein at 21 kDa, dot blot hybridization confirms same result as shown in Fig. 7.

Discussions

Several steps had been taken in the effort of overcoming hepatitis infection for years; include forcing of a new prophylaxis vaccine and effective treatment curing HBV infection. Major concept of this research is to fulfill the need of the possibility doing immune therapy concept in the future by producing combination of some antigen as an alternative therapeutic treatment in combating HBV infection. As a part of those major aims, this research taking apart in preparing highly expressed HBcAg protein suitable for clinical treatment, in which expressing hepatitis core antigen (HBcAg) protein in L. lactis.

We were using NZ3900 L. lactis strain for expression. This strain is a standard strain to express various proteins that is critical to be produced in a food grade strain of L. lactis family. Since it has deletion on lacF gene, thus it will not depend on lactose level for gene expression, conversely it leans on the Nisin concentration. Addition of usp45 at the -N terminal of the construct will force secretion of produced protein. Schematic representation gene construct as shown in the Fig. 1 shows that usp45-HBcAg gene was placed after Ncol restriction enzyme at the -N terminal. Furthermore, the placement of gene of interest behind nisin inducible promoter (PnisA) initiates auto-induction of the gene expression.

The HBcAg gene, which was expressed in this research, is in the form of codon optimised suitable for lactic acid bacteria’s expression. We have previously been informed that optimisation of codon is not always leads to improvement on protein expression. Several factors involved, such as environment condition during protein expression and the cell cycle. However, the manual instruction of expression system using nisin-controlled genes (NICE-MoBiTech GmbH), mentioned a requirement of specific codon usage to increase the protein product. Approximately, around 35–37% GC content of the DNA is required for lactic acid bacterial expression. We calculated the inserted gene’s GC contents for both native and adapted sequences. It shows that actual codon adaptation increase the GC contents for about 2% from total sequence, it raises from 45.8% (with 253 of GC amino acids out of 552 amino acids in total) to 47.8% (with 263 of GC amino acids out of 552 amino acids in total). It is quite above the range of GC content suggested by the NICE manufacturer. However, since both native and adapted sequence are under 50% GC, it seems that the L. lactis conveniently expressing the gene, it was supported by the amount of total protein raise for about 10–20% (Fig. 5A).

To determine the difference of expression level, codon optimised clone was compared with non-optimised form (native). Slot blot method was used to monitor protein bands and Image J software (Institute of Health, USA) available for download at: http://rsb.info.nih.gov/ij was performed to quantify the bands. The quantification was not absolute value but reflect the relative amounts as a ratio of each protein band. Three various conditions (5, 10, and 50 ng/ml of inducer) are observed, while the highest expression level of codon optimised is obtained at 50 ng/ml of Nisin inducer. Another study reported that recombinant protein with NICE system could express higher protein level by added 5 or 10 ng/ml of Nisin [23, 24]. Furthermore, the purified protein was also successfully confirmed through dot blot hybridization and western blotting techniques (Fig. 7).

There are only few reports confirm the ability of L. lactis bacteria in handling genes in high GC content (more than 50% of GC), one of the example is expression A. vinelandii genes that having 63–67.5% [25], membrane proteins [26], human papilloma virus E7 antigen [27], chitine gene [28] and some others. However, most of those genes are in the native form, not being optimised avoiding higher codon biases to be expressed in L. lactis. This research even sharpens the potential owned by L. lactis in expressing various genes. The improvement by optimising the codon of the inserted gene could be valuable in increasing the protein production. Our results could be promising not only intensifying the use of L. lactis for more broadly use expression host, but also accommodating the needs of HBcAg antigen for therapeutic treatments. Future improvements will be needed.
to increase expression that possibly done through insertion of some propeptide residues [29], in other way measurements of secreted protein is in a correct folding through in vivo testing might be crucial.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References


