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Heterologous Expression of Interferon α-2b in *Lactococcus lactis* and its Biological Activity against Colorectal Cancer Cells

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Type I Interferons (IFNα) are known for their role as biological anticancer agents owing to their cell-apoptosis inducing properties. Development of an appropriate, cost-effective host expression system is crucial for meeting the increasing demand for proteins. Therefore, this study aims to develop codon-optimized IFNα-2b in *L. lactis* NZ3900. These cells express extracellular protein using the NICE system and Usp_{45} signal peptide. To validate the mature form of the expressed protein, the recombinant IFNα-2b was screened in a human colorectal cancer cell line using the cytotoxicity assay. The IFNα-2b was successfully cloned into the pNZ8148 vector, thereby generating recombinant *L. lactis* pNZ8148-SP_{Usp45}-IFNα-2b. The computational analysis of codon-optimized IFNα-2b revealed no mutation and amino acid changes; additionally, the codon-optimized IFNα-2b showed 100% similarity with native human IFNα-2b, in the BLAST analysis. The partial size exclusion chromatography (SEC) of extracellular protein yielded a 19 kDa protein, which was further confirmed by its positive binding to anti-IFNα-2b in the western blot analysis. The crude protein and SEC-purified partial fraction showed IC_{50} values of 33.22 μg/ml and 127.2 μg/ml, respectively, which indicated better activity than the metabolites of *L. lactis* NZ3900 (231.8 μg/ml). These values were also comparable with those of the regular anticancer drug tamoxifen (105.5 μg/ml). These results demonstrated *L. lactis* as a promising host system that functions by utilizing the pNZ8148 NICE system. Meanwhile, codon-optimized usage of the inserted gene increased the optimal protein expression levels, which could be beneficial for its large-scale production. Taken together, the recombinant *L. lactis* IFNα-2b is a potential alternative treatment for colorectal cancer. Furthermore, its activity was analyzed in the WiDr cell line, to assess its colorectal anticancer activities in vivo.

Keywords: **Colorectal cancer, IFNα-2b, *Lactococcus lactis*, pNZ8148, recombinant protein, WiDr**

Introduction

Colorectal cancer (CRC) is the third common type of disease in men and women with 10% and 9.4% of the total cases worldwide, respectively. Its occurrence in the developed regions were almost 60% of the whole cases [1]. Meanwhile, Indonesia is one of the developed countries with emerging CRC incidence, and currently at the fourth ranks in the top ten, by age-standardized and mortality rates [2]. Presently, the utilization of 5-fluorouracil (5-FU) in various cancer treatments is known to

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evoke survival, while the largest effect was produced in the CRC type, both in weekly and daily injections. The overall response of 5-FU in the advanced CRC was limited to 10–15%, therefore, the addition of irinotecan and oxaliplatin increase survival rate, however, elevated toxicity [3, 4].

Type I Interferons (IFNα) are pleiotropic cytokine encoded by at least 13 IFNα genes possessing many functions, i.e., antiviral, antitumor, anticancer, and immune regulator [5]. The recombinant of IFNα-2b (rhIFNα-2b) is a glycoprotein, which being the first therapeutic protein approved by the USFDA (United State Food and Drug Administration). As many as 86 countries use it for hepatitis and cancer treatments, including for hairy cell leukemia, renal cell carcinoma, melanoma, AIDS-related Kaposi's sarcoma (KS), follicular lymphoma, and chronic myelogenous leukemia [6]. IFNα-2b has been used as monotherapy, and also combined with other drugs for cancer treatment, such as 5-FU, tamoxifen, and vinblastine [3]. The combination treatment of tamoxifen and IFNα-2b for estrogen-positive breast cancer (MCF-7) and liver cancer (HepG2) cell, produced inhibition towards cell growth [7]. Moreover, the purified human IFNα enhances the growth-inhibitory effect of 5-FU in the hepatocellular carcinoma (HCC) cell line PLC/PRF/5 [8]. Therefore, the requirements of substantial amount of rhIFNα-2b for cancer treatments are highly needed, as well as therapeutic protein and the development of biopharmaceutical products. Moreover, Indonesia still imports hIFNα, and according to the Indonesian Ministry of Health, the strategic plan on biopharmaceutical development is by stimulating domestic production of the biosimilar product, including IFNα that is expected to be executed in 2019–2021 [9–11].

Several studies have reported hIFNα-2b production strategies in various vectors, including *Escherichia coli, Saccharomyces cerevisiae, Pichia pastoris, Streptomyces lividans,* mammalian cells, plant nuclear genome, and chloroplast [12]. Since the single treatment of IFNα-2b was short-lived, many studies also reported the conjugation of IFNα-2b with various fusions, i.e., monomethoxy polyethylene glycol (PEG) and Human Serum Albumin (HSA), that generated more stability and improved the protein half-life [13–15]. As the protein production using this host expression system is found to be costly, alternative methods that entail low cost are still being studied. One of the gram-positive and food-grade bacteria which is appointed with a GRAS (Generally Regarded as Safe) status by the USFDA is *Lactococcus lactis*. The engineering of *L. lactis* has been developed to be a vehicle of protein, DNA, or antigen, and used for numerous intracellular or extracellular protein productions without any inclusion bodies. The other advantage of using *L. lactis* as a protein expression cell factory is the absence of lipopolysaccharide (LPS) or any proteases, which is in contrast to those of *E. coli* and *Bacillus subtilis*. The recombinant protein produced in *L. lactis* gave the best production yields with secretions up to five-fold higher than that of cytoplasmic system [14, 16].

Currently, there is still a limited report on recombinant IFNα-2b with codon-optimized in the food-grade host system. Previous studies have been focusing on codon-optimized HBcAg synthetic gene in food-grade host system, and resulting in 1.5–3.2 folds of protein expression level [17]. This study reports the codon-optimized IFNα-2b in *L. lactis* as a component of live therapeutic protein. Furthermore, its activity is analyzed against WiDr cell line, due to the limited data on colorectal anticancer property.

Material and Methods

Bacterial Strains and Culture Conditions

The bacterial strains and plasmids used in this study were shown in Table 1. *E. coli* was grown on Luria Bertani (LB) with aeration at 37℃, while *L. lactis* was cultured in M17 broth supplemented with 0.5% glucose at 30℃ without shaking. Antibiotics were added into the medium for each culture as follows: ampicilin (100 μg/ml) for *E. coli* strain, chloramphenicol (10 μg/ml) for recom-

Table 1. Bacterial strains and plasmids used in this study.

Strains and Plasmids	Charactersitics	Source
E. coli MC1061	Cloning host strain	MobiTec
L. lactis NZ3900	Expression host strain	MobiTec
pMA-T	Amp^r; cloning vector	Invitrogen
pNZ8148	Cmp^r; nisin inducible promoter; *L. lactis* expression vector	MobiTec

Abbreviations: Amp^r, Ampicillin resistance; Cmp^r, Chloramphenicol resistance.

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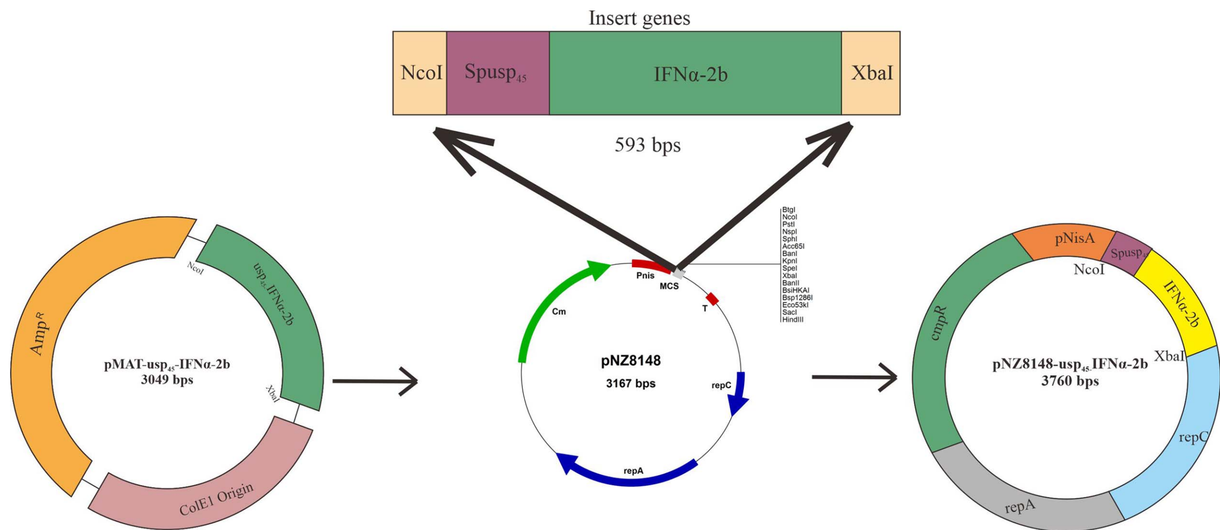

Fig. 1. Representation of the pNZ8148-SP$_{Usp45}$-IFNα-2b construct map. The recombinant plasmid containing insert gene IFNα-2b and SP$_{Usp45}$ was concatenated and ligated into pNZ8148 expression vector.

binant *L. lactis*. Agar plates made by the addition of 1.5% (w/v) bacteriological agar (Himedia) to the liquid medium.

Design and Sequence Analysis of Codon Optimized IFNα-2b Synthetic Gene

The human IFNα-2b (hIFNα-2b) synthetic gene (accession number: DQ922943) was codon-optimized by the Integrated DNA Technologies Inc. USA (Suppl. 1). It was found that codon optimization affected greatly the up-regulation of protein and RNA levels [18]. The synthetic gene has already been adjusted to two base pair -CC-, to allow translation of fusion at *Nco*I (CCATGG) site [19], and attached into pMAT vector. The two restriction enzymes, *Nco*I and *Xba*I, were introduced at the -N and -C terminals, respectively (Fig. 1). The sequence of codon-optimized IFNα-2b was analyzed using Geneious Prime® software 4.8.5. Alignment was carried out using native sequence as the reference. The amino acid sequence was determined using the TRANSLATE tool and the pI value on Expasy. The tertiary structure of codon-optimized IFNα-2b was predicted using Phyre^2 Server (http://www.sbg.bio.ic.ac.uk/phyre2). To determine the disulfide bond of the tertiary structure, the Geneious Prime® software was used to obtain the S-S position of the protein.

Cloning and Construction of pNZ8148-SP$_{Usp45}$-IFNα-2b

The recombinant pMAT-IFNα-2b was digested with 40 U/μl endonuclease restrictions of the *Xba*I and *Nco*I (Biolabs Inc™). The fragment of digestion product was purified using a QIAquick Gel Extraction Kit (Qiagen, USA), and ligated into pNZ8148 vector with 3 U/μl T4 ligase at 4℃ overnight. The IFNα-2b gene used in this study was 498 bps in length, and the extracellular protein expression utilized was 81 bps long of the signal peptide (SP$_{Usp45}$; GeneBank M60178.1) in the coding sequence (Fig. 1). The transformant colonies were selected on the LB agar containing selectable marker chloramphenicol. The recombinant was confirmed by the PCR colony using P*nisA*_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. The PCR conditions in the 35 cycles were as follows: pre-denaturation at 94℃ for 3 min, denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min, elongation at 72℃ for 30 sec, followed by a final elongation at 72℃ for 6 min [20].

Transformation of Recombinant IFNα-2b into Expression Vector *L. lactis* NZ3900

The recombinant pNZ8148-SP$_{Usp45}$-IFNα2b were selected and transformed into *L. lactis* NZ3900 using a Gene Pulser Apparatus (Bio-Rad, USA). The 10% of overnight growth cultures in M17 media supplemented with 0.5% glucose was inoculated into fresh media containing 0.5% (w/v) glucose, 0.5 M sucrose, and 2.5% (w/v) glycine and incubated on 30℃ until reached OD_{600} = 0.3.

Furthermore, the culture was centrifuged 6000 ×*g* for 10 min at 4℃, and the pellet was resuspended with washing buffer (0.5 M sucrose, 10% glycerol). The resuspended pellet was centrifuged for the second time, and then diluted using the same buffer with addition 10 mM EDTA, followed by incubation on ice for 15 min. Samples were centrifuged 6000 ×*g* for 10 min at 4℃ and diluted on 1 ml wash buffer. Recombinant plasmid was transformed into competent cells through electroporation 2000 V, 25 µF, 200 Ω, followed by addition of 400 µl fresh media. After that, the transformants were selected on agar media containing 10 µg/ml of chloramphenicol. Selected colonies carrying insert gene were verified using promotor nisA and terminator pNZ8148 primers [20]. The DNA sequence analysis of the construct were undertaken at the First Base Laboratories (Malaysia), while the sequence similarity were carried out with BLAST analysis (https://blast.ncbi. nlm.nih.gov/Blast).

Expression and Purification of Recombinant IFNα-2b Protein

The recombinant *L. lactis* NZ3900 consists of pNZ8148- SP_{Usp45}-IFNα-2b cultivated on M17 media, supplemented with 0.5% glucose, and added with 10 µg/ml chloramphenicol. The recombinant strains cultures were induced with 10 ng/ml nisin when the strains grow at OD_{600} ~ 0.5. The supernatant were harvested by centrifugation on 10,000 rpm at 4℃ for 30 min. The protein precipitation used 65% (w/v) ammonium sulphate $[(NH_4)_2SO_4]$ at 4℃ overnight. The protein pellets were harvested by centrifugation and suspended in 100 mM Tris-HCl pH 8. A Size Exclusion Chromatography (SEC) purification method was performed by aqueous solvent known as gel filtration. The loading protein samples were added into the pre-equiblirated sephadex G-50 pre-packed coloumn (GE Healthcare^{TM}, USA). The elution protein samples were fractionated by 100 mM Tris-HCl (pH 8) with a 1 ml/min flow-rate. Protein fractionation were collected and analyzed with spectrophotometer at 280 nm [17].

Tricine SDS-PAGE and Immunoblotting Analysis

Protein validation was analyzed using Tricine SDS-PAGE for final acrylamide concentration at 14%, and stained with Silver Stain Kit (Pierce, USA). The protein was loaded with Tricine Sample Buffer (Novex, Thermo, USA) by 1:2 ratios, respectively. Protein detection was carried out by immunoblotting in accordance with the standard protocol described previously [21]. Protein fusions in polyacrylamide gel were transferred into nitrocellulose membrane, and hybridization signals were detected using polyclonal anti-mouse IFN-2b (Abcam, USA) at a dilution of 1:1000. The Horse-Peroxidase anti-mouse IgG (Promega) was used to detect specific binding. The protein-antibody bindings were visualized using BCIP/NBT substrate (Thermo Scientific, USA).

Total Protein Quantification

The IFNα-2b concentration was determined using Bichinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific, USA). The standard protein used bovine serum albumin (BSA) in serial dilution of 2 mg/ml to 0.025 mg/ml. The working reaction was prepared by mixing Reagent A and B with 50:1 and loaded into 96-well plates. The samples were added by 1:20 for triplicate. The plates were incubated at 37℃ for 30 min and absorbance was measured at 570 nm using ELISA reader (Thermo Fisher Scientific).

Biological Activity of Purified IFNα-2b in Colorectal Cancer Cell Line (WiDr)

The anticancer activity of the purified IFNα-2b in colorectal cancer cell line (WiDr) using a MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) method. The protein obtained in the SEC purification was freeze-dried, and suspended in DMSO. The WiDr cells were obtained from Cancer Chemoprevention Research Center (CCRC) of Gadjah Mada University. It was cultured on 10 cm/10 ml petri dish in a complete medium (RPMI, 10% FBS, 1% penicillin-streptomycin), incubated at 37℃, 5% CO_2 up to 80% confluent, and then harvested using cells harvesting protocol. The confluent cells were checked and counted on *Zoe Cell Imager* (BioRad, USA). The cells were washed using 3 ml steriled PBS 1×, followed by the addition of 500 µl *Trypl*-E and incubated at 37℃, 5% CO_2 for 3 min. Furthermore, the cells were harvested by the addition of 2 × 2 ml complete medium, then cultured into 96-well plate (10^4/well) and incubated for 24 h. The cells were washed using PBS 1x before treated with samples. The partial purified IFNα-2b was diluted into 50, 100, 200 and 400 µg/ml

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concentration using complete medium. The treated cells were incubated for 24 hrs, their images after treatment were checked on *Zoe Cell Imager* (BioRad), and followed by the addition of 0.5 mg/ml MTT solution. The plate was incubated for 3 hrs and the cytotoxic assay was stopped by addition of stopper solution containing SDS 10% and HCl 1 M. The absorbance was measured in 570 nm ELISA *Microplate Reader* (Multiscan, Thermo) [22]. The data (Means ± SD) was carried out in the percentage of the untreated controls. The concentration required for 50% inhibition of cells viability (IC_{50}) was defined using Graphpad Prism® software. Statistical analysis was performed by ANOVA with significant difference at $p < 0.05$.

Results

Design and Computational Analysis of Codon-Optimized IFNα-2b Sequence

The sequence alignment using Geneious Prime® software generated changes about 28% and expressed in *L. lactis* (shown by red circle in Fig. 2). The GC contents of codon-optimized IFNα-2b genes were represented as 41.9% (shown by green circle on Fig. 2). *L. lactis* has average GC content of 35.4%, and was reported to secret foreign protein up to 20 mg/l with optimized gene construct [23]. The tertiary predicted structure of codon-optimized IFNα-2b has five α-helices (A-E) (Fig. 3). The helices are connected by a loop, AB, BC, CD, DE. As shown in Fig. 3, the four cysteine residues (Cys1-Cy98, Cy29-Cy138) which formed two disulfide bonds were observed, and also the five free cysteine residues were

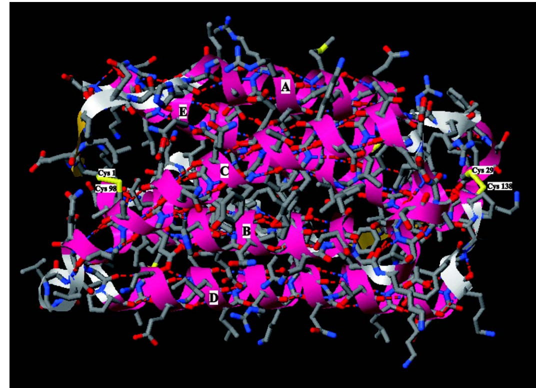

Fig. 3. Predicted 3D structure of codon-optimized recombinant IFNα-2b using Phyre2 Server. The helices are starting from N-terminal, which are containing five alpha helices (A-E). Two disulfide bonds were pointed in yellow bonds for Cys 1-Cys 98 and Cys 29-Cys 138. Free cysteine residues were in single yellow atom color.

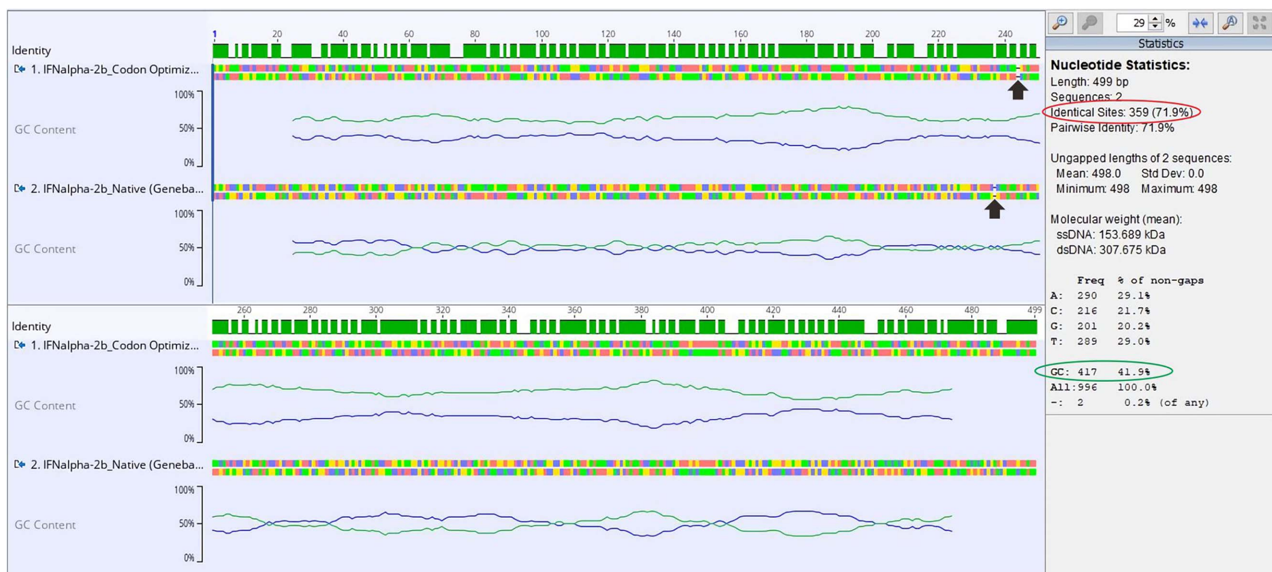

Fig. 2. Sequence alignment of codon-optimized and native IFNα-2b used Geneious Prime® software. Line graph represented base contain, GC graph was pointed by blue line, and AT graph in green line. Gaps were found on different places (shown by black arrows). Geneious Prime® software allowed to quantified GC contents percentage of codon optimized sequence (shown by green circle), which showed the amount of A, C, G, T, U, S, W nucleotides in the sequence, foremost G, C or S.

pointed in yellow atom color. This result confirmed that codon optimization of IFNα-2b does not affect the tertiary protein structure, which is crucial in biological activity. The different 3-dimensional predicted IFNα-2 had been reported, in which IFNα-2b structure was mentioned in five and seven helices. Based on the five helices model, the AB loop (residue 22–38) exhibited structural diversity, which is part of receptor binding segment and involved in the interaction to its cellular receptor. The biological conformations were kept by disulfide bond of Cys29 and Cys138. The residues within loop AB and helix E assisted most of the extracellular interaction of IFNAR2 receptor [12, 24].

Construction of Recombinant IFNα-2b Expressed in *L. lactis* NZ3900

This study showed the codon-optimized IFNα-2b gene, ligated into pNZ8148 vector of *L. lactis* NZ3900, utilizing nisin inducible promoter. The host expression system with inducible promoter relied on recombinant protein production. The electrophoresis confirmed the presence of recombinant construct of pNZ8148-SP$_{Usp45}$-IFNα-2b

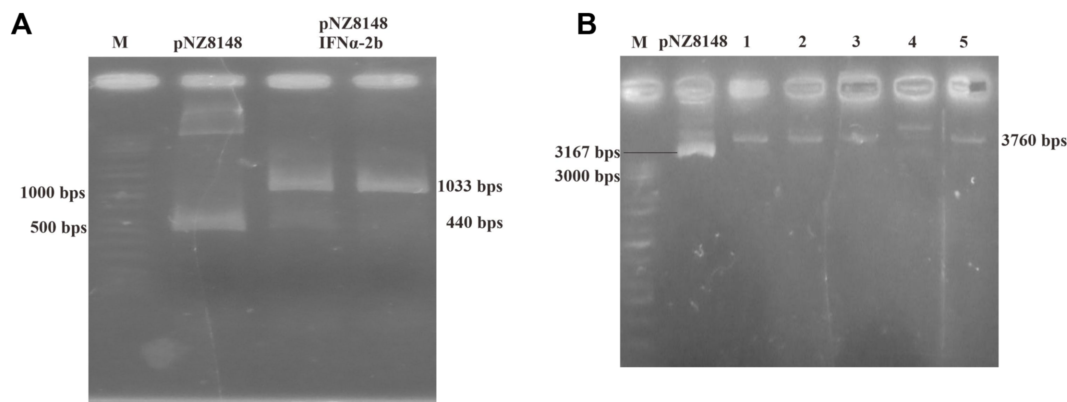

Fig. 4. Validation of DNA plasmid construct in *L. lactis* recombinant IFNα-2b. (A) DNA plasmid PCR product electrophoregram used F/R PnisA and TpNZ8148 primers; (B) electrophoregram of isolated recombinant plasmid from transformant colonies 1-5. M: DNA marker of 100 bp (Vivantis).

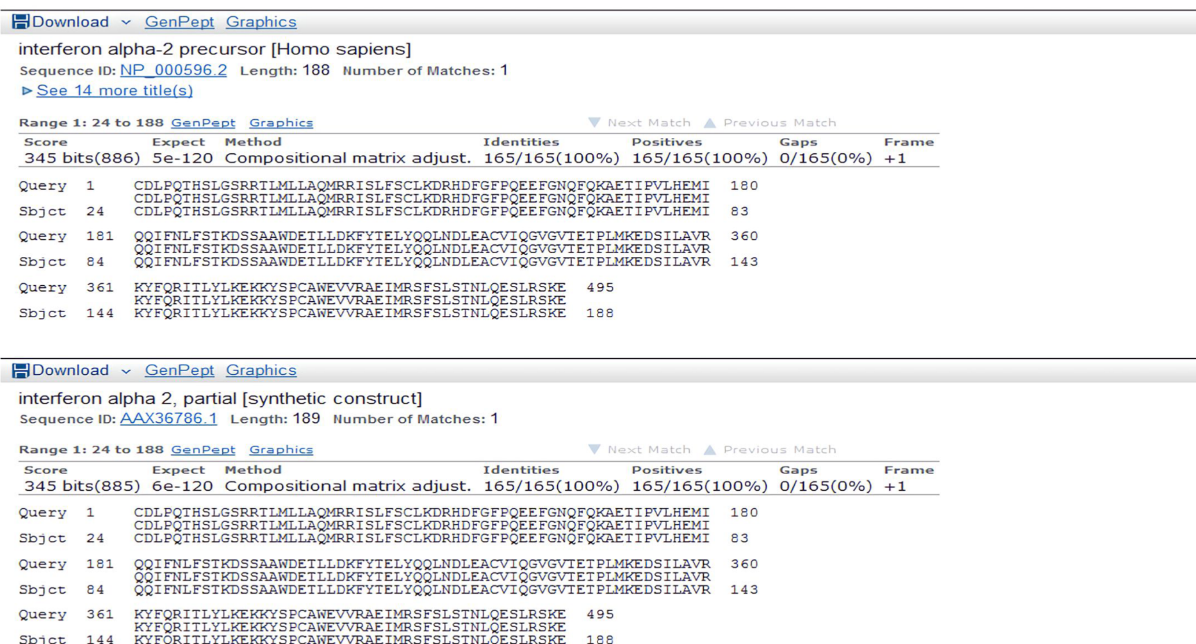

Fig. 5. BLAST tool confirmation of codon-optimized IFNα-2b amino acid sequence was 100% identical to human IFNα-2b.

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which generated amplicon of ±1033 bps, and empty plasmid (pNZ8148) of ±440 bps (Fig. 4). The recombinant pNZ8148-IFNα-2b was 3760 bps containing 593 bps length of SP_{Usp45}-IFNα-2b. The IFNα-2b gene was successfully transformed into multi-cloning site in the pNZ8148 expression vector. The plasmid pNZ8148 is one of the common vectors used in NICE system. It has a derivative of the high-copy number plasmid pSH71 [25], and does not contain the *nisK* and *nisR* genes. Therefore, the application has to use *nisRK* derivative strain, such as *L. lactis* NZ3900. The combination use of plasmid and host expression vector was very important in obtaining optimum recombinant system. The high expression level by using the *L. lactis* NZ900/pNZ8148 combination have been reported in >800-fold on the reflection of Brazzein [26]. In addition, the aligned amino acid sequence of codon-optimized IFNα-2b with the accession number of AAX36786.1, was confirmed 100% similar to the origin human IFNα-2b (Fig. 5). Therefore, the result validated that the construction of recombinant pNZ8148-IFNα-2b was successfully transformed into *L. lactis* NZ3900, and did not induce any mutation of insert gene.

Heterologous Expression, Purification, and Characterization of Recombinant IFNα-2b Protein

Heterologous protein was obtained in the addition of SP_{Usp45} which encoded major extracellular protein in *L. lactis*. The SP_{Usp45} ordered the protein secretion into the medium, and was declared efficiently to raise protein

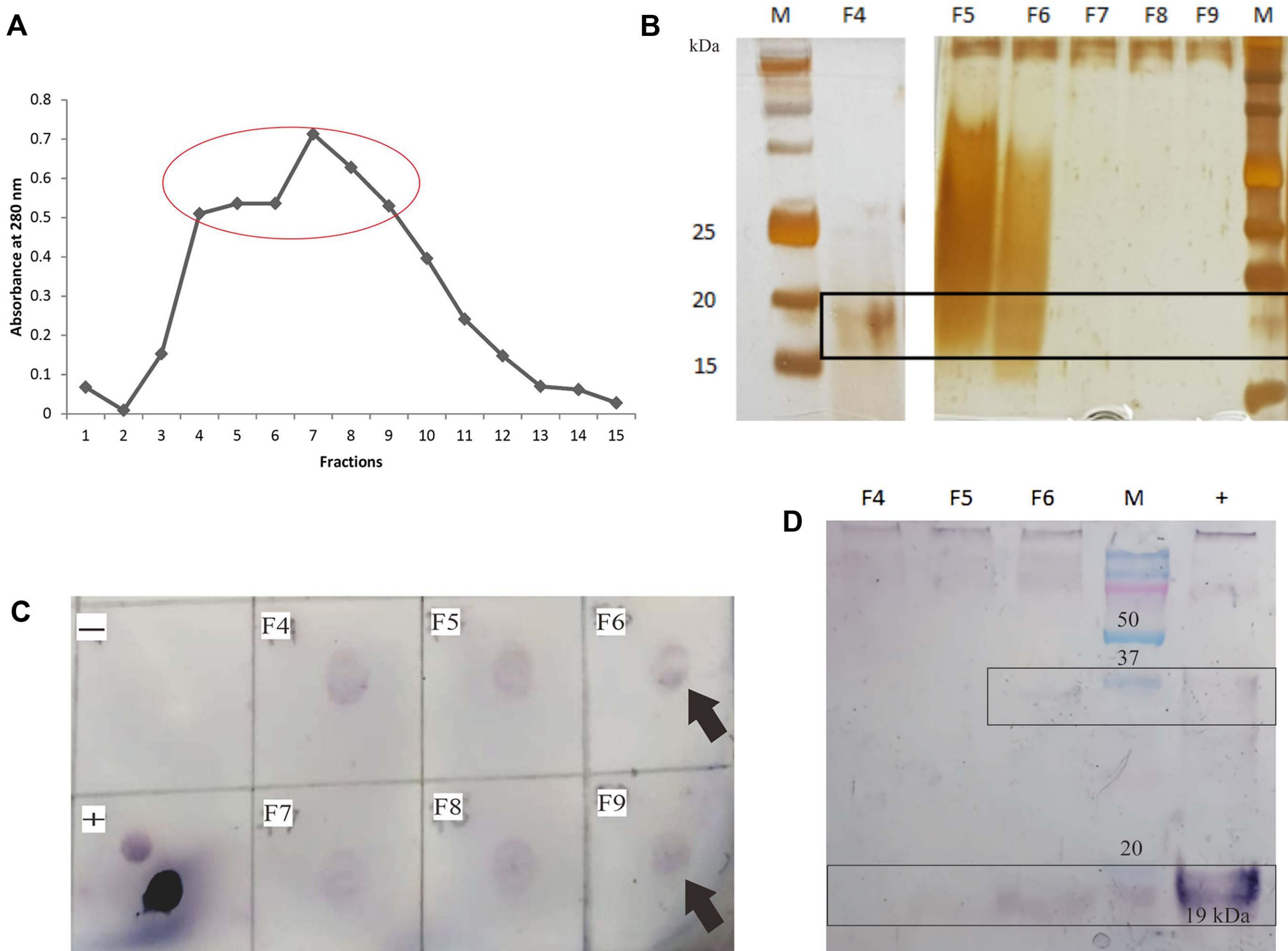

Fig. 6. Chromatogram profile of Size Exclusion Chromatograph (SEC) IFNα-2b. (A) Absorbance of IFNα-2b measured at 280 nm; (B) Tricine SDS-PAGE of partial fraction 4-9; (C) Dot-blot hybridization of confirmed SEC fraction; (D) Western blot analysis of partial fraction 4-6 of recombinant *L. lactis* pNZ8148-SP_{Usp45}-IFNα-2b; (+) control of rhIFNα-2 (Biovision # P01563).

expression of bacteriocins, enterocin, and hiracin 1.5–3.6 fold higher [27]. The soluble form of recombinant IFNα-2b in the partial SEC fraction were measured at 280 nm, and the absorbance of partial fraction 4–9 was given as average protein of A_{280} 0.5–1.5 range (Fig. 6A) [28]. The partial protein fraction was corroborated in the Tricine SDS-PAGE bearing a band of 19 kDa for F4, F5, and F6 (Fig. 6B). The Dot-blot analysis also gave a positive hybridization for fraction 4–9, which was slighter signal on the F7–F9 (Fig. 6C). Contrastingly, the partial fraction of F7–F9 was not detected in any band of Tricine SDS-PAGE, however, it gave a slight signal in the Dot blot which was assumed as terribly low IFNα-2b in the sample. The further analysis for Western-blot was carried out by using the fraction that had already been confirmed as target protein in the SDS-PAGE and Dot blot (F4–F6). As shown in Fig. 6D, there were clear band at 19 kDa for F5 and F6, which was a slighter signal on F5 than F6, however, both of the fractions were confirmed as target protein which was in line with positive control of IFNα (Biovision #P01563). Contrastingly, there was no band detected in F4. Moreover, there was a slight band at ~37 kDa of F6 and the control. The protein that appeared at ~37 kDa corresponded to the aggregation formed and still bioactive. Although, it appeared at ~37 kDa which is larger than expected, the western blot analysis confirmed the positive signal hybridization with anti-IFNα-2b. This result confirmed that the expressed protein both in 19 kDa and ~37 kDa was in the mature form. Furthermore, some evidence suggested that the aggregated form of rhIFNα-2b was obtained through the formation of reducible linkages (disulfide bonds). It was also suggested that the failure of SDS and mercaptoethanol to dissolve aggregates was due to the inability to access the interfere protein molecules formed by the strong hydrophobic interactions [29]. In addition, the disulfide bridge was essential to be retained, since it plays important role in the biological activity. In this case, buffers seem to play a significant role in maintaining protein stability. Tris-HCl pH 8.0 buffer was used to stabilize protein, and most of the protein stability in solution was above the pI value. As known, the codon-optimized IFNα-2b had the isoelectric point (pI) value of 5.97 (Supplementary Data 2). The rhIFNα-2b stability reports in various buffers had been suggested that the aggregation was induced with reduced condition, after storage 15 days, which generated a dimer detected band on the SDS-PAGE profile [29]. Therefore, it was assumed that the slight aggregated form of IFNα-2b was due to certain condition, such as the storage time, which drove the protein instability similar to the previous report. Furthermore, it was also assumed that the presence of the positive and negative ion charges within the buffer interacted with protein residues (charge-charge interaction). Moreover, free cysteine residues were easily oxidized [29, 30], since the codon-optimized IFNα-2b has five free cysteine residues (Fig. 3). From the data obtained, the heterologous IFNα-2b expressed in *L. lactis* NZ3900, with pNZ8148 containing SP_{Usp45} still compromised a success result and well-

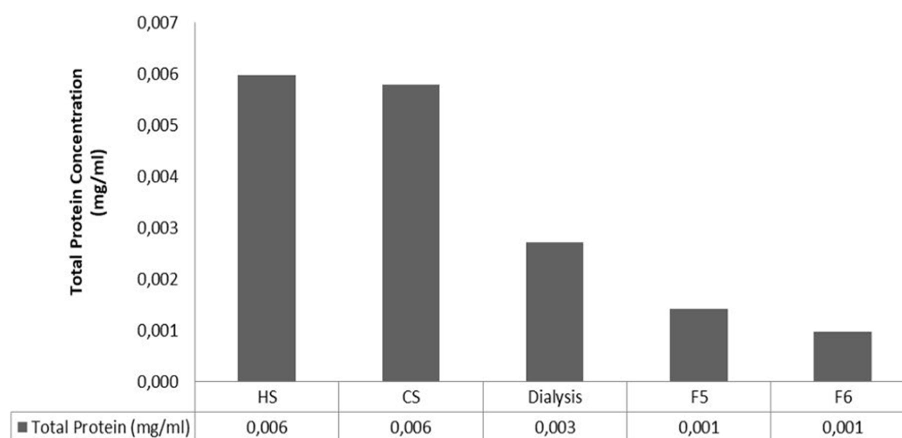

Fig. 7. Total protein quantification of IFNα-2b in every step of purification using BCA Kit Assay. Culture Supernatant (CS), Host (*L. lactis* NZ3900) Supernatant (HS), partial purified fraction 5 and 6 (F5-F6).

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characterized as its native molecule, since the aggregates formed was only detected in slight signal.

Furthermore, the concentration of protein expressed was determined by using the BCA assay which represented the total protein concentration in the purification steps. The Culture Supernatant (CS) has the highest concentration, which is normally quantified in the abundant volume (Fig. 7). The host *L. lactis* NZ3900 supernatant (HS) and the CS concentration are not different, since the heterologous protein expressed in *L. lactis* was identified in eight genes (*ffh*, *lspA*, *secA, secE, secG, secY, sipL, tig*) [23]. This data indicated that CS contains another protein expressed in the host, and still need more purification steps to obtain the target type, since the BCA assay only quantified, and not the specific protein. Furthermore, the total protein concentration graph declined in the purification steps. The lowest concentration was obtained in the SEC partial fraction, volume (1 ml), which was lower than than that of CS and dialysis samples.

Anticancer Activity of Recombinant IFNα-2b Against Colorectal Cancer Cell Line (WiDr)

The capability of this recombinant protein was proven to have anticancer activity represented on the human colorectal cancer cell line (WiDr). The cytotoxicity effect was discovered by MTT methods. The principle of this method was the reduction of tetrazolium salts into formazan crystal. This was undertaken in the living cells which implicated the mitochondrial system, while apoptosis cells were affected by drug activity that did not perform MTT reduction. Therefore, any increase and decrease of viable cell was measured by formazan concentration depicted by optical density [31, 32]. The percentage of cell viability was obtained from the cell numbers which were not inhibited. This represented cell growth inhibition of drug and reflected the sensitivity, which was specified as the concentration to achieve 50% growth inhibition (IC_{50}) [32].

The WiDr viability percentage and the IC_{50} determination were shown in Table 2. All samples treatment affected the viability cells gradually, and most produced the best effect at 400 µg/ml. The CS/IFNα-2b metabolites inhibited 50% WiDr growth at 33.22 µg/ml, which were in line with the high protein concentration, rather than other samples. The elevated IC_{50} value in every purification step of recombinant IFNα-2b, was caused by the decreasing concentration of the protein present on each sample. The purified partial fraction of SEC generated the best IC_{50} value at 127.2 µg/ml, compared to the host's metabolites *L. lactis* NZ3900 which has higher IC_{50} value at 231.8 µg/ml. Referring to total protein quantification, HS and CS have slight difference and generate significant difference cytotoxicity effect on WiDr cells. It was observed that most of the protein quantified in CS was IFNα-2b, in mature structure for biological activity. Furthermore, in the control treatment, Tamoxifen produced IC_{50} value of 105.5 µg/ml, which is better than purified IFNα-2b. The purification processes used for obtaining the target protein affected the cytotoxicity result in the cell line. Another purified recombinant IFNα-2b was reported using AKTA purifier 10 system utilizing His trap column (GE Healthcare), and generated the highest inhibition on the MCF-7 cells at 4 µg/ml [7]. While IFNα-2b expressed in *P. pastoris* inhibited 44% breast cancer (MCF) cell proliferation at 20 nmol/L

Table 2. The cytotoxic effect in WiDr cell viability and IC_{50} determination.

Samples	% Cell Viability				IC_{50}
	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)	400 (µg/ml)	
CS	17.846 ± 1.323^{a}	19.035 ± 0.651^{a}	15.029 ± 2.905^{a}	-22.654 ± 3.053^{a}	33.22
Dialysis	82.729 ± 4.676^{b}	43.710 ± 0.564^{b}	10.732 ± 0.651^{a}	0.000 ± 0.426^{ab}	90.56
F5	73.916 ± 6.764^{bc}	68.301 ± 7.933^{c}	81.592 ± 10.58^{b}	33.831 ± 0.015^{c}	127.2
F6	78.607 ± 3.467^{b}	76.333 ± 7.541^{cd}	71.500 ± 9.711^{b}	26.084 ± 6.946^{bc}	159.8
HS	86.780 ± 14.63^{b}	87.491 ± 1.420^{cd}	78.891 ± 1.919^{b}	14.286 ± 1.128^{bc}	231.8
Tamoxifen	84.967 ± 20.231^{b}	93.229 ± 15.39^{d}	69.709 ± 27.53^{b}	65.861 ± 24.951^{d}	105.5

CS: Culture Supernatant, HS: Host (*L. lactis* NZ3900) Supernatant. Significantly difference at $p < 0.005$ shown in different subset of same coloumn numbers.

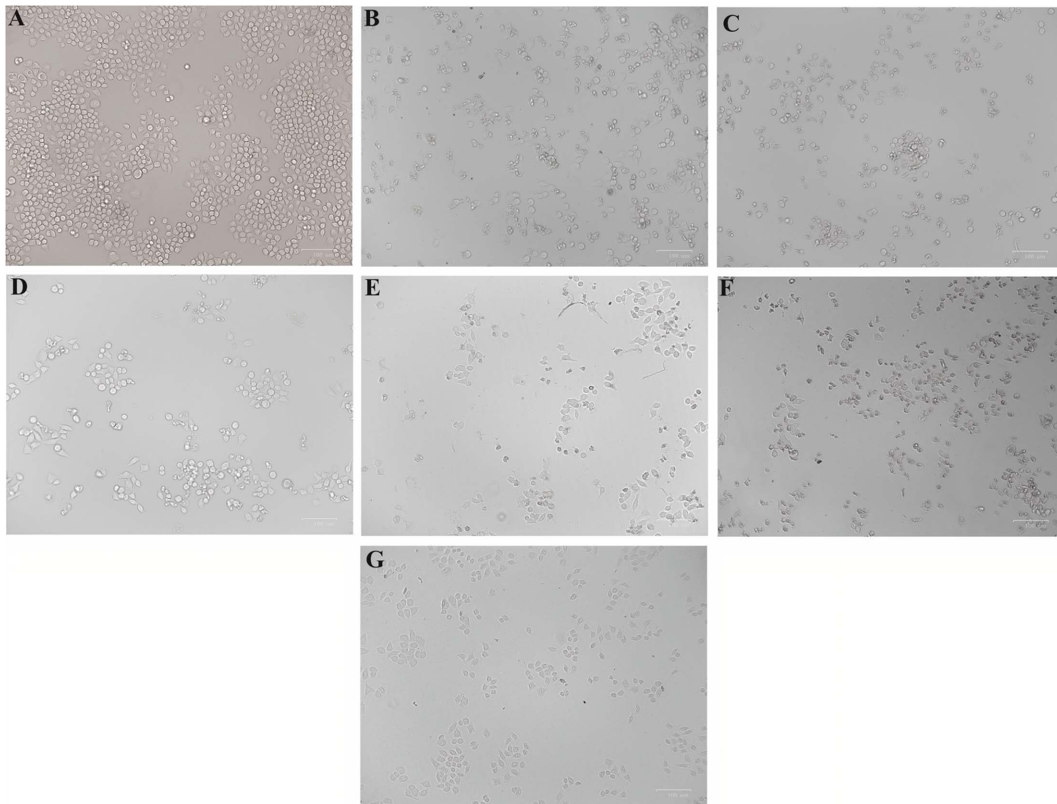

Fig. 8. WiDr cells morphology after samples treatment at 400 μg/ml. (A) Untreated cells; (B) treated with CS, (C) dialysis; (D) F5; (E) F6; (F) cells treated with HS; (G) positive control of treated cells with tamoxifen.

[33]. The IC_{50} value of 5-fluorouracil (5-FU) as a drug for colorectal cancer was reported at 129.29 μg/ml [34]. The result showed that a cytotoxicity of purified IFNα-2b was also comparable with the commercial drug.

The cytotoxic effect in WiDr cells morphology was shown in Fig. 8. The cells image with the highest samples concentration treatment was compared with the significant cytotoxic effect of each sample to the untreated cells. The treatment with the highest sample concentration of 400 μg/ml affected apoptosis in most of the treated cells, which was significantly shown in Fig. 8B, 8C, and 8F. The original morphology of the untreated WiDr cells was displayed mostly in the healthy round shape, with consistent size, and cohesive interaction with other cells (Fig. 8A). However, the treated cells appeared smaller, breakage interaction with other cells, and membrane damaged. These biological difference shapes as compared to the untreated cells extensively represented the apoptosis. The apoptotic cells morphology was also described to become shrunken with nuclear condensation, cellular blebbing, refracted, and development of the body formation in human colorectal cancer (HT-29) cell line [35]. The mechanism of action in cytotoxic effect of IFNα-2b, by inducing apoptosis was reported through elevating p53 response, and activating p28 in the stress signaling response to cell death, and also triggering caspase pathway [36].

Discussion

Several strategic steps have already been reported in overcoming CRC in Indonesia, including systematic therapy with adjuvant which induced immune system [37]. IFNα was a type I cytokine which have been established as immunotherapy agent in cancer treatment. The hIFNα-2b was a glycoprotein, consisting of 166 amino acids, each monomer contains five α-helices, which preserved the molecule integrity. IFNα-2a and IFNα-2b were type I cytokine produced by recombinant technology, and both differ by a single amino acid at

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position 23 [38]. In challenging biopharmaceutical development action, the production of applicable therapeutic protein engineering in the future should be low cost, and mostly in the purification procedure. This research majorly focused on preparing highly expressed IFNα-2b in *L. lactis* and meeting the protein demands.

The codon-optimized IFNα-2b was expressed in *L. lactis* NZ3900. This host strain is a food grade and a progeny of NZ3000, which was developed for food grade application. This strain had been reported to be used for clinical test approach, secreting therapeutic protein, such as PRRSV [39]. Since this strain has a deletion of *lacF,* which is operon lactose integrated into the chromosome, for the expression level to depend on the inducer concentration. The derived high-copy number plasmid pNZ8148 containing *nisA* promoter with *nisK* and *nisR* genes was used as recombinant vector that could form a two-component regulatory system to activate promoter. The existence of nisin in the medium was detected by NisK, a histidine-protein kinase that reposed in the cytoplasmic membrane, and was proposed to act as a receptor for the mature nisin molecule. The nisin in the medium generated autophosphorylation of cluster gene, and leading to auto-induction of expression process on the insert gene downstream in the MCS, when inserted into the pNZ8148 vector (Fig. 1) [40]. Furthermore, 10 ng/ml nisin was applied to optimize the heterologous expression level. Previous studies reported the construction of bacteriocins from various origins (plantaricin E, F, and W), which were successfully expressed in *L. lactis,* generating mature form at transcription and translational level [19, 41, 42]. Another studies also reported that nisin concentration at 10 ng/ml enhanced protein expression level 4.59x of native HBcAg gene [20], and conversely codon-optimized HBcAg gene was reported to produce the highest expression level with 50 ng/ml nisin concentration [17].

The synthetic IFNα-2b gene was optimized to raise the expression level, while codon optimization is widely used for recombinant protein expression. The basis approach of this method was to improve codon usage by increasing its frequency in native genes coding sequences, that have high protein expression levels. The codon usage for heterologous genes in *L. lactis* should have GC contents about 35–37% (NICE-Mobitec, GmbH). The codon-optimized IFNα-2b sequence analysis showed the GC content of 41.9%, which was slightly above the GC content range (Fig. 2). The increasing GC content of codon-optimized gene also conveniently expressed HBcAg gene in *L. lactis,* with the total protein raises of 10–20% compared to the native gene [17]. Moreover, the expressed target protein was one of the crucial factors in obtaining the expected yields. The SP_{Usp45} was fused with N-terminal and translocated into the medium. Several studies focused on the comparison of protein yields and cytoplasmic production. The quantification of various protein (Nuc, E7, IFNω, L7/L12) in the secreted form was higher than that of the cytoplasmic [43, 44]. Extracellular expression of recombinant protein in the food grade bacteria is a promising approach, which reduces the purification cost and refolding steps for clinical application. The intracellular expression of IFNα-2b in *L. lactis* had been reported with induction of 30 ng/ml nisin, and showed bioactivities of 1.9×10^6 IU/mg [45]. However, it required the costly purification procedure. In addition, the combination of *L. lactis* NZ3900 and pNZ8148 vector confirmed the appropriate expression system that generated IFNα-2b in the mature form. Since the previous studies have reported a precursor form of IFNα and IFNβ expressed in both supernatant and cellular samples, which also applied the NICE expression system *L. lactis* NZ9000/pNZ8048, and *L. lactis* NZ9000/pSec [46–48]. Furthermore, this study also defines the superior potential of SP_{Usp45} attached in the appropriate plasmid vector (pNZ8148) than some other extracellular signal peptide, and also promote the enhancement of expression for scaling up [26].

Subsequent to the western blot analysis that generated the aggregates form of IFNα-2b, the biological activity assay was used to confirm the bioactivity. The mature form was tested in the colorectal cancer cell line (WiDr). The MTT converting process of viable cells with active metabolism generated a purple coloured formazan product which accumulated as an insoluble precipitate inside the cells, as well as being deposited near the cell surface and in the medium [49]. The culture supernatant of IFNα-2b gave the best cytotoxic effect rather than other samples (Table 2), and the treated cells with IFNα-2b induce cell apoptosis (Fig. 8). This data confirmed that IFNα-2b in the CS samples was in the mature form, although it was detected in the aggregates type. Proteins are charged amphiphilic polymers which self-associate

easily, possessing naturally reversible oligomers of different sizes, depending on the association tendency, concentration, and other solution condition. Many proteins have been shown to have reversible oligomers, and their interaction has also been identified as electrostatic, which lead to the formation of reversible, native dimers, trimmers, and oligomers [50]. In conclusion, this study showed an approach in providing delivery system vector, expressing well-characterized rhIFNα-2b which is applicable as live therapeutic agent, and also suggested for clinical test in future research. This recombinant vector system can be used in large scale production for meeting protein demands. Further improvement is also needed to optimize purification method from *L. lactis* with purifier tools, such as AKTA, gene expression level by qRT-PCR, and molecular cytotoxic study in the cell line by FACS. Moreover, the quantification of proteins, secreted by both *in vitro* and *in vivo* testing is also required.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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