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CODON OPTIMIZATION AND CO-EXPRESSION OF THERMOSTABLE β-GALACTOSIDASE AND L-ARABINOSE ISOMERASE IN *LACTOCOCCUS LACTIS* FOR SINGLE-STEP PRODUCTION OF FOOD-GRADE D-TAGATOSE

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ABSTRACT : D-Tagatose is a promising low-calorie sucrose substitute having prebiotic properties. In this study, biosynthesis of D-tagatose was achieved by constructing and expressing a polycistronic plasmid encoding codon-optimized thermostable B-galactosidase from *Marinomonas* sp. BSi20414 and L-arabinose isomerase from *Clostridium hylemonae*, simultaneously, in *Lactococcus lactis* NZ3900. Both enzymes were expressed as soluble and functional proteins under control of the nisinA inducible promoter and the optimum concentration of nisin was found to be 1 ng/ml. The induced cells were immobilized in chitosan beads and used for D-tagatose production from lactose as a whole-cell biocatalyst. Results revealed an efficient bioconversion of lactose to D-tagatose and maximum production was accomplished at 60°C after 12 h, starting with 20% (w/v) lactose. The recombinant cells entrapped in chitosan beads converted up to 34% of lactose into D-tagatose under optimum conditions in a single step that could be implemented in safe-production of food-grade low-calorie sweetener.

Key words : Immobilization, chitosan beads, optimization, D-galactose, bioconversion.

INTRODUCTION

Nowadays, the demand for healthy low-calorie sweeteners is growing, especially amongst people with type 2 diabetes, cardiovascular diseases and obesity. D-Tagatose is a naturally existing rare ketohexose that considered aspromising sucrose replacement (Torrico *et al*, 2019). It has almost equal sweetening power as sucrose but with less than one-third the caloric value of the latter, thus suggested as a low-calorie sweetener (Levin, 2002). Not only the low-calorie merit, but D-tagatose is also a free radical scavenger (Mooradian *et al*, 2019) having prebiotic properties (Jayamuthunagai *et al*, 2017). Furthermore, D-tagatose has been recently described as a potential nutraceutical (Roy *et al*, 2018). Although, it can be produced chemically by direct isomerization of D-galactose with metal hydroxides, the drastic reaction conditions and high cost of the subsequent purification steps have limited the application of the chemical method (Guo *et al*, 2018). On the other hand, enzymatic production is regarded as an environmentally friendly alternative. Enzymatically, D-tagatose can be produced using L-arabinose isomerase (EC 5.3.1.4) by isomerization of D-galactose (Zheng *et al*, 2017; Zheng *et al*, 2019; Bober and Nair, 2019). L-arabinose isomerases derived from various microbial sources including *Enterococcus faecium*, *Lactobacillus brevis*, *L. plantarum* and *Geobacillus kaustophilus* have been identified to be ion-dependent enzymes requiring Mn^{2+} and/or Co^{2+} as cofactors (Choi *et al*, 2016; Jayamuthunagai *et al*, 2017; Guo *et al*, 2018; de Sousa *et al*, 2019). In spite of their activity, the ion-dependent nature is unfavorable for the food industry owing to the toxicity of Co^{2+} (Xu *et al*, 2014). Recently, a novel ion-independent thermostable L-arabinose isomerase originating from *Clostridium hylemonae* has been described (Nguyen *et al*, 2018). The isomerization at high temperature is thought to shift the bioconversion equilibrium to D-tagatose production, reduce the viscosity of the reaction mixtures, and abate the opportunity of microbial contamination. For cost-effective production of D-tagatose, D-galactose could be produced enzymatically from the relatively cheap sugar, lactose. B-galactosidase (B-D-galactoside-galactohydrolase, E.C. 3.2.1.23) catalyzes the hydrolysis of glycosidic bond between a terminal non-reducing â-D-galactoside unit and an aglycone moiety (Juers *et al*, 2012). Naturally, itcatalyzesthe hydrolysis of its lactoseto glucose and galactose andfound in a wide variety of microorganisms (Xia *et al*, 2018; Xu *et al*, 2019; Aburto *et al*, 2019; Rutkiewicz *et al*, 2019). Lately, a novel thermostable B-galactosidase originated from

Marinomonas sp. BSi20414 with promising activity retaining a considerable activity after prolonged incubation at elevated temperature has been described (Ding *et al*, 2017). The goal of this study is the cost-effective production of D-tagatose in a single step from lactose by co-expression of synthetic thermostable B-galactosidase and L-arabinose isomerase originated from *C. hylemonae* and *Marinomonas* sp. BSi20414, respectively, in *Lactococcus lactis*. Notwithstanding *L. lactis* is regarded as an important workhorse for protein expression, codon usage of heterologous genes frequently biased from the host. Consequently, codon optimization of the desired genes is thought imperative to accomplish high expression levels (De Waele *et al*, 2018; Solopova *et al*, 2019; Duan *et al*, 2019; Mohseni *et al*, 2019). The present investigation emphasizes on rational design of a co-expression system using the synthetic biology approach to create D-tagatose biosynthetic pathway in an engineered strain of *L. lactis*.

MATERIALS AND METHODS

Sequence retrieval and *in silico* analysis

The B-galactosidase sequence of *Marinomonas* sp. BSi20414 (Accession number A0A1L1YPL4) and L-arabinose isomerase sequence of *C. hylemonae* (Accession number EEG73787) were retrieved from the GenBank (NCBI). The prediction of signal peptide was conducted by Signal P 5.0 server (Almagro Armenteros *et al*, 2019). The molecular weight, theoretical PI, aliphatic index and other physicochemical properties were predicted using the Protparam tool (https://web.expasy.org/protparam/).

Codon optimization and synthesis of chimeric DNA

The codon usage of the two open reading frames (ORF) were optimized *in silico* for expression in *Lactococcus lactis* and synthesized at IDT (Integrate DNA Technology, Inc., USA). The ORF of B-galactosidase gene was followed by Shine-Dalgarno (SD) sequence (AGGAGGTATATAC) properly spaced from the start codon (ATG) of L-arabinose isomerase gene by 8 nucleotides as aligned spacing (AS). The restriction sites of NcoI and SacI were inserted at the 52 and 32 ends of the synthesized chimeric DNA fragment, respectively.

Construction of polycistronic plasmid

The synthetic DNA cassette was digested with NcoI and SacIand cloned into the equivalent restriction sites of the pNZ8149 expression vector (MoBiTec, Germany) under the control of the inducible nisinA promoter (*PnisA*). Afterwards, the recombinant polycistronic plasmid was transformed into *L. lactis* NZ3900 cells by electroporation. The transformants exhibiting yellow colonies on Elliker agar (HiMedia, India) supplemented with 0.5% lactose were selected.

Nisin induction and co-expression

The transformed *L. lactis* NZ3900 were grown statically at 30°C in M17 broth (Merck, USA) supplemented with 0.5% glucose until an optical density (OD) at 600 nm of 0.4. Afterwards, nisin was added (0.1-3 ng/mL) to induce the co-expression of B-galactosidase and L-arabinose isomerase genes for 3.0 h. Then cells were harvested by centrifugation at 15,000 rpm for 20 min at 4°C. The induced cell pellet was then washed with phosphate buffered saline (PBS) and disrupted by sonication. Cell debris was removed by centrifugation at 15,000 rpm for 30 min at 4°C and the supernatant was collected and assayed for B-galactosidase and L-arabinose isomerase activity.

B-galactosidase assay

The activity of B-galactosidase was detected by chromogenic substrate ortho-nitrophenol-B-D-galactopyranoside (ONPG) assay (Ding *et al*, 2018). The release of ortho-nitrophenol (ONP) was monitored by measuring the absorbance at 420 nm. The concentration of ONP was obtained from the standard curve. One unit of B-galactosidase activity was defined as the amount of enzyme that catalyzes the liberation of 1 umol ONP per minute. Protein concentration was assayed by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

L-arabinose isomerase assay

L-arabinose isomerase activity was assayed at 60°C for 30 min by using100 mMD-galactose as a substrate in 25 mMTris–HCl buffer (pH7.5) containing 100uM $Mn^{2+}$and 50 uM Co^{2+} (Hung *et al*, 2014). The amount of the produced D-tagatose was determined by using the cysteine-carbazole sulfuric acid method (Dische and Borenfreund, 1951). Briefly, the enzyme reaction was terminated by adding 900 uL of 9 M sulfuric acid into the 100 uL reaction mixture. Then 30 uL of 100 mM cysteine hydrochloride was added that followed by 30 uL of 10 mM carbazole solution. After incubation for 30 min, the absorbance was read at 560 nm to estimate D-tagatose concentration against the linear standard curve prepared by D-tagatose. One unit of L-arabinose isomerase activity was defined as the amount of enzyme required to produce 1 umol of keto-sugar in 1 min.

Whole-cell permeabilization and immobilization

The induced cell pellets were resuspended in POM buffer (50 mMK_2HPO_4, 50 mMKH_2PO_4 and 1

mMMgCl_2, pH 7.4). To kill and permeabilize the cells, an equal volume of absolute ethanol was added and incubated at room temperature for 30 min (Yu and O'Sullivan, 2014). These permeabilized cells were washed with ice-cold POM buffer and resuspended in 2% low-molecular-weight chitosan (50–190 kDa; 75–85% deacetylated) in 1% acetic acid. The produced mixture was then added dropwise into a solution of 1.5% sodium triphosphate with vigorous stirring. After stirring for 1 h at room temperature, the formed chitosan beads were washed with distilled water and served as immobilized whole-cell biocatalyst (Yu and O'Sullivan, 2018).

D-Tagatose production by the induced *L. lactis* whole cells

Permeabilized cells containing the recombinant thermostable B-galactosidase and L-arabinose isomerase were used as a biocatalyst to produce D-tagatose directly from lactose. The whole-cell biocatalyst immobilized in chitosan beads were incorporated in 50 mM phosphate buffer (pH 6.5) containing 100mM lactose and incubated at 60°C for 30 min with stirring (150 rpm). Subsequently, cells were collected by centrifugation at 6000 rpm for 20 min, and the concentration of D-tagatose was determined in the supernatant the cysteine-carbazole sulfuric acid method.

Influence of reaction conditions on D-tagatose production

To determine the optimum temperature for D-tagatose production, the immobilized biocatalyst was incubated in 50 mM phosphate buffer (pH 6.5) containing 100mM lactose at various temperatures (30–90°C). After 30 min, the concentration of the produced D-tagatose was determined. The optimal pH was determined by assaying D-tagatose concentration after incubation invarious buffer systems (pH range of 4.0–9.0). The used buffers were: 50 mM sodium acetate buffer (pH 4.0–5.5), 50 mM phosphate buffer (6.0 to 7.5) and 50 mM Tris–HCl buffer (pH 8.0–9.0). To evaluate the impact of metal ions, the bioconversion of lactose to D-tagatose was assessed in the presence of 1 mM of $CaCl_2$, $ZnCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$ and $NiCl_2$. The effect was expressed as a relative conversion (%) that was determined by considering 100% activity of the reaction without the addition of metal ions (control).

Influence of lactose concentration on D-tagatose production

The immobilized whole-cell biocatalyst beads were incorporated in 50 mM phosphate buffer (pH 6.5) containing 100-500 g/L lactose and incubated at 60°C with stirring (150 rpm). Afterwards, cells were collected by centrifugation at 6000 rpm for 20 min and the concentration of D-tagatose was determined in the supernatant using the cysteine-carbazole sulfuric acid method.

Influence of incubation period on D-tagatose production

The optimum incubation period was determined by incorporation of the immobilized whole-cell biocatalyst beads in 50 mM phosphate buffer (pH 6.5) containing 200 g/L lactose and incubated at 60°C with stirring (150 rpm). Samples were withdrawn at regular 2h intervals for 18 h and the concentration of D-tagatose was determined in the supernatant. The productivity was expressed as a relative conversion (%).

Statistical analysis

Data are expressed as the mean ± standard deviation. Significant differences among the treatments were determined by one-way analysis of variance (ANOVA) and subsequently by Duncan's multiple range test in IBM SPSS Statistics (version 21.0, IBM Corp., USA). Statistical significance was indicated at a confidence level of 95%.

RESULTS

Sequence retrieval and *in silico* analysis

The B-galactosidase sequence of *Marinomonas* sp. BSi20414 (1971bp), coding for 656 amino acid residues, and L-arabinose isomerase sequence of *C. hylemonae* (1506bp), coding for 501 amino acid residues were retrieved from the GenBank. According to the Signal P 5.0 server, the two proteins lack signal peptides and are predicted to be intracellular proteins. The physicochemical properties computed by Protparam analysis revealed the thermal stability potential of both investigated proteins. The expected molecular weight, theoretical PI and aliphatic index were calculated (Table 1).

Codon optimization and synthesis of chimeric DNA

To improve the heterologous co-expression of B-galactosidase and L-arabinose isomerase in *L. lactis*, the ORF of each sequence was optimized by substituting the rare codons and reducing the GC content; to match that of the host. Regarding B-galactosidase, GC content decreased from 48.21% of the wild type to 36.29%, which is close to the level for optimal expression in *L. lactis*. Similarly, the GC content of L-arabinose isomerase was optimized from 51.59% to 34.93%. Moreover, the SD sequence was designed downstream the stop codon of the optimized B-galactosidase sequence to initiate the expression of its downstream gene (L-arabinose isomerase) that spaced by a proper AS. To assure the

Table 1 : Physicochemical parameters of β-galactosidase and L-arabinose isomerasecomputed using ExPASy's ProtParam tool

Parameter	β-galactosidase	L-arabinose isomerase
Number of amino acids	656	501
Molecular weight	74.271 kDa	56.554 kDa
Theoretical isoelectric point (pI)	5.84	5.20
Total number of negatively charged residues (Asp + Glu)	81	76
Total number of positively charged residues (Arg + Lys)	62	56
Grand average of hydropathicity (GRAVY)	-0.321	-0.314
Aliphatic index	82.13	82.20

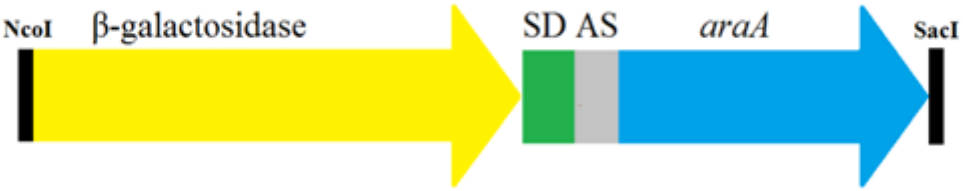
cloning direction, NcoI and SacI restriction sites were incorporated at the 52 and 32 ends of the designed sequence, respectively. The optimized sequence was synthesized and the chimeric DNA fragment was obtained (Fig. 1).

Co-expression of B-galactosidase and L-arabinose isomerase

The codon-optimized synthetic DNA cassette was inserted into pNZ8149 vector and transformed into the food-grade *L. lactis* NZ3900 cells harboring the nisin-controlled gene expression system (NICE). The co-expression was induced by nisin for 3 h and then cells were harvested and sonicated. Results revealed a successful functional expression of the two genes. The influence of nisin concentration on the expression level of both genes was investigated and the optimum concentration of nisin for co-expression was found at 1.0 ng/mL (Fig. 2). At the optimum concentration of nisin, B-galactosidase was expressed in an active form with a specific activity of 48 U/mg protein while the specific activity of L-arabinose isomerase was estimated to be 57.3 U/mg protein. At higher concentrations of nisin, a significant decrease in the specific activity of B-galactosidase was observed. Regarding L-arabinose isomerase, the maximum activity was found when cells were induced with 1.5 ng/mL nisin. However, a further increase in the inducer concentration results in a sharp drop in the enzyme activity.

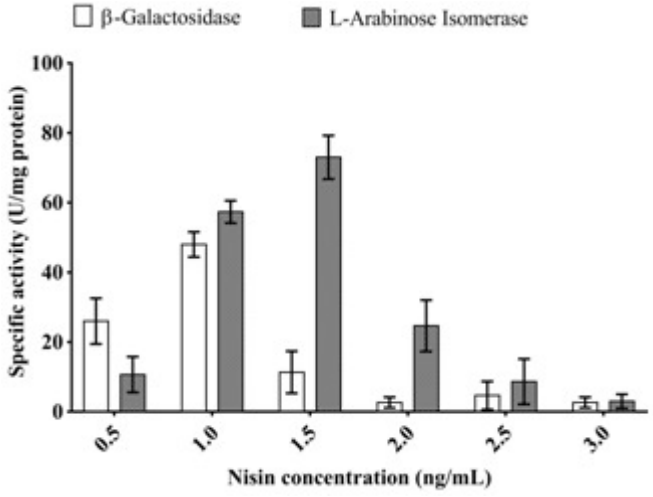
Bioconversion of lactose to D-tagatose

Concerning D-tagatose production by immobilized recombinant biocatalyst, results clarified the simultaneous production and isomerization of D-galactose to produce D-tagatose in a single step starting with lactose as a substrate. The maximum production of D-tagatose was achieved at 60-70°C (Fig. 3A) over a wide range of pH ranging from 6.8 to 8.0 (Fig. 3B). Interestingly, the presence of Mg^{2+} magnified the D-tagatose yield by more



- SD = Shine-Dalgarno sequence, AS = Aligned spacing

Fig. 1 : Schematic diagram of the co-expression construct.

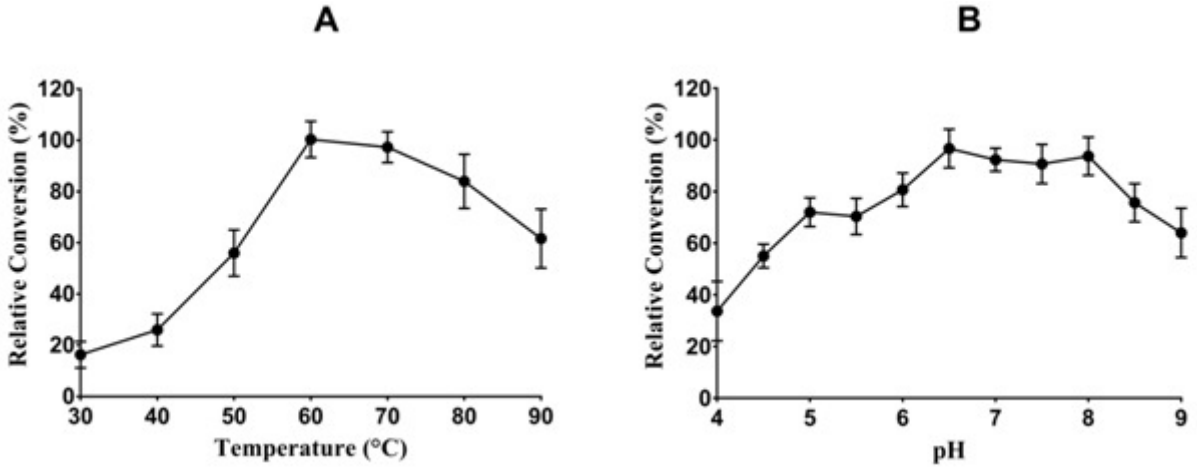


- Error bars represent standard deviations (SD).

Fig. 2 : Influence of nisin concentration on expression levels.

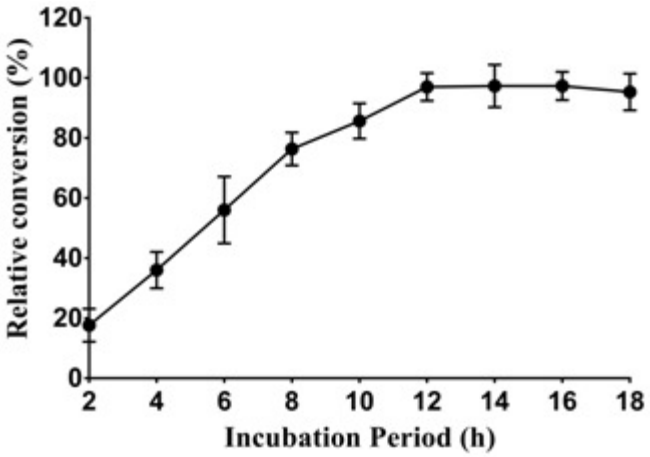
Table 2 : Influence of different metal ions on D-tagatose production. The effect of each metal ion was investigated at 1mM. D-tagatose conversion in control samples was set as 100%. Relative conversion values that take the same letter, indicates that there is insignificant difference between these treated groups according to Duncan's multiple range test ($p<0.05$)

Chemical Effector	Relative conversion (%)
Control	100^{b}
Ca^{2}	97.45 ± 5.21^{b}
Zn^{2+}	104.12 ± 6.12^{b}
Mg^{2+}	135.60 ± 13.87^{a}
Mn^{2+}	109.76 ± 12.33^{b}
Co^{2+}	107.60 ± 11.81^{b}
Cu^{2+}	43.17 ± 8.40^{d}
Ni^{2+}	77.54 ± 17.53^{c}



- Error bars represent standard deviations (SD).

Fig. 3 : Effect of temperature (A) and pH (B) on D-tagatose production.



Error bars represent standard deviations (SD).

Fig. 4 : Effect of incubation period on D-tagatose production.

than 35%compared with control (Table 2).Impact of initial lactose concentration on the biotransformation process was investigated. Increasing initial lactose concentration results in a direct increase in D-tagatose yield, yet the maximum conversion rate was achieved at 20% lactose (Table 3). Further increase in lactose concentration causes a significant reduction in conversion percentage. Moreover, the effect of incubation period on D-tagatose production was assessed. Results indicated a progressive increase in conversion percentage with time and the plateau was perceived at 12 h (Fig. 4). Ultimately, the immobilized recombinant biocatalyst converted 34.8% of lactose to D-tagatose after 12 h incubation at 60°C starting with 200g/L lactose in 50mM phosphate buffer (pH 6.5).

Table 3 : Impact of lactose concentration on D-tagatose yield.

Lactose concentration (g/L)	D-Tagatose Yield(g/L)	Conversion (%)
100	26.3	26.3
200	69.6	34.8
300	73.2	24.4
400	78.9	19.7
500	86.5	17.3

DISCUSSION

The present investigation addresses a novel eco-friendly approach to produce food-grade D-tagatose from lactose in a single step by co-expression of codon-optimized thermostable B-galactosidase and L-arabinose isomerase genes in a probiotic strain. The choice of B-galactosidase from *Marinomonas* sp. BSi20414 and L-arabinose isomerase from *C. hylemonae* is based on their admirable characteristics (Ding *et al*, 2017; Nguyen *et al*, 2018). Notwithstanding the availability of few reports describing D-tagatose production utilizing recombinant technologies, the vast majority reported D-tagatose production by employing *Escherichia coli* as a host (Xu *et al*, 2016; Patel *et al*, 2017; Manzo *et al*, 2019). Although, *E. coli* is the first option for the expression of prokaryotic proteins, some safety and technical concerns may restrict its application in food-related products. These concerns include risks due to endotoxins formation; as well as the technical production issues regarding the formation of inclusion bodies, the two-membrane nature of the cell wall and the relatively complicated aerobic cultivation conditions (Makrides, 1996; Mierau *et al*, 2005). On the other hand, *L. lactis* is a food-grade generally recognized as safe (GRAS) bacterium in which no endotoxins or inclusion bodies are formed and requires simple non-aerated cultivation conditions (Song *et al*, 2017; Berlec *et al*, 2018). Before designing the expression cassette, we retrieved sequences of both genes and performed *in silico* characterization. Due to the absence of signal peptides at their N-terminals, both were

classified as intracellular proteins. Thus the complete ORFs from the start codon to the stop codon were determined to be subjected to the subsequent design attempts. The predicted intracellular localization agreed with previous reports on bacterial B-galactosidase and L-arabinose isomerase (Men *et al*, 2012; Sun *et al*, 2018). *In silico* analysis revealed an excess of negatively charged residues over positively charged ones, indicating their maintained stability and solubility through preferential hydration (Kyne *et al*, 2017). Besides, the calculated aliphatic indicesby Protparam were more than 82 suggesting the thermal stability of both enzymes. Likewise, elevated aliphatic index of proteins has been described as an indication of their thermostability(Shukla *et al*, 2018). Based on the *in silico*-gathered data, a rational design was adopted to optimize codons of both genes. In this process, the GC content of coding sequences was reduced to resemble that of the host cells and rare codons were replaced by synonymous codons that consistent with *L. lactis* codon-usage. It has been believed that biased usage of synonymous codons adapted to tRNA pools has a strong effect on protein expression due to balanced tRNA supply and demand that accomplishes optimal translation (Quax *et al*, 2015; Parret and Besir, 2016). Furthermore, the SD sequence was inserted as a ribosomal binding site located 8-bases upstream of the start codon of L-arabinose isomerase to initiate the expression of the latter and constructing a polycistronic expression sequence. Previous reports indicated that the distance between the SD sequence and the start codon have fluctuated from 5 to 12 nucleotides, though, the optimal aligned spacing length regarding gene expression level was 8 nucleotides (Tauer *et al*, 2014). Herein, both genes of interest were successfully co-expressed as functional soluble enzymes in the food-grade strain *L. lactis* NZ3900. The crucial role of nisin concentration on expression level has been appraised. Being an inducer, nisin concentration controlled the level of expression. Being an inducer, nisin concentration has a direct impact on gene expression. At its fittest concentration (1 ng/mL), both enzymes were more or less expressed at a comparable level nevertheless further increase hurt enzyme activity. The adverse consequence may be due to excessive overexpression of the protein that could contribute to improper protein folding or aggregation of insoluble proteins (Nguyen *et al*, 2018).The induced cells then were immobilized in chitosan beads and served as a whole-cell biocatalyst to produce D-tagatose utilizing lactose as a substrate. Immobilization is thought to be an efficient means to boost the biocatalyst stability and implement successive using and recycling of the biocatalyst (Jin *et al*, 2016).The induced recombinant cells co-expressing B-galactosidase and L-arabinose isomerase converted hydrolyzed lactose to glucose and galactose that in turn is converted to its isomer, D-tagatose, by the action of L-arabinose isomerase. Results showed obvious stability and elevated operating temperature of chitosan-entrapped whole-cells regarding D-tagatose production at 60-70°C. This value is higher than the optimal temperature of various L-arabinose isomerases derived from *Enterococcus faecium* (Manzo *et al*, 2019), *Pseudoalteromonas haloplanktis* (Xu *et al*, 2016), and *Bacillus licheniformis* (Zhang *et al*, 2009). Though, extra-stable *Anoxybacillus flavithermus* L-arabinose isomerase catalyzing the isomerization at a temperature of 95° has been reported (Li *et al*, 2011). In agreement with our findings, the remarkable improvement of D-tagatose production imposed by Mg^{2+} is also documented in a recent investigation (Nguyen *et al*, 2018). On the other hand, D-tagatose production in most previous studies was enhanced in the presence of Co^{2+} and Mn^{2+} (Patel *et al*, 2016; Du *et al*, 2019). Results revealed that the maximum conversion rate of lactose to D-tagatose was achieved at 20% (w/v) initial lactose concentration and the conversion rate decreases by a further increase in lactose concentration. The negative impact exerted by higher lactose concentration could be due to the transgalactosylation activity of B-galactosidase. It has been reported that various B-galactosidase possess transgalactosylation activities at higher substrate concentrations producing galacto-oligosaccharides (Kittibunchakul *et al*, 2019; Vera *et al*, 2019; Botvynko *et al*, 2019; Xin *et al*, 2019). Thus at higher concentrations of lactose, a significant portion of the produced galactose could be consumed in the transgalactosylationreaction instead of the isomerization to D-tagatose. In this investigation, up to 34.8% of the initial lactose was converted to D-tagatose at a single step using the food-grade recombinant *L. lactis* harbouring and expressing B-galactosidaseand L-arabinose isomerase. Hence, this study explored an efficient single-step production of the functional sweetener, D-tagatose, from a relatively cheap substrate, lactose, using engineered probiotic strain.

CONCLUSION

The current work established a promising co-expression system for single-step production of D-tagatose from lactose by constructing a polycistronic plasmid encoding codon-optimized B-galactosidase and L-arabinose isomerase in the food-grade *L. lactis* NZ3900. The recombinant cells entrapped in chitosan beads converted up to 34% of lactose into D-tagatose

under optimum conditions in a single step that could be implemented in safe-production of low-calorie sweetener.

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