Current Research in Food Science 4 (2021) 646–654

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Vitamin B12 biofortification of soymilk through optimized fermentation with extracellular B12 producing *Lactobacillus* isolates of human fecal origin

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ARTICLE INFO

Keywords:
Isolation
Lactobacillus reuteri
Lactobacillus rhamnosus
B12 Biofortification
Soymilk
OFAT-GSD-ANFIS optimization
Extracellular vitamin B12
Infant fecal sample
cbiK gene

ABSTRACT

The present study was designed to bio-fortify the soymilk (*per se* a B12-free plant food matrix). The PCR-based screening characterized the human fecal samples (4 out of 15 tested) and correspondingly identified novel lactobacilli isolates (n = 4) for their B12 production potential and rest (n = 62) as negative for this attribute. Further, 3 out of the 4 selected strains showed ability for extracellular vitamin production. The most prolific strain, *Lactobacillus reuteri* F2, secreted B12 (132.2 ± 1.9 μg/L) in cobalamin-free-medium with the highest ratio ever reported (0.97:1.00; extra-: intra-cellular). In next stage, the soymilk was biofortified *in situ* with B12 during un-optimized (2.8 ± 0.3 μg/L) and optimized (156.2 ± 3.6 μg/L) fermentations with a ~54-fold increase at Artificial Neuro Fuzzy Inference System based R value of >0.99. The added-nutrients, temperature and initial-pH were observed to be the most important fermentation variables for maximal B12 biofortification. We report *Lactobacillus rhamnosus* F5 as the first B12 producing (101.7 ± 3.4 μg/L) strain from this species. The cyanocobalamin was extracted, purified and separated on UFLC as nutritionally-relevant B12. Besides, the vitamin was bioavailable in an auxotrophic-mutant. The lactobacilli fermentation is suggested, therefore, as an effective approach for B12 biofortification of soymilk.

1. Introduction

Vitamin B12 is well known for its ability to play a vital role in the human physiology and its deficiency may cause peripheral arterial diseases, megaloblastic anemia, cardiovascular and neurological disorders (Nielsen et al., 2012). Microorganisms, free living or animal gut associated, are the sole producers of B12 that become available to humans through consumption of animal tissues and related food-products. A recent report suggested a high prevalence (up to 46%) of B12 deficiency in a developing country like India (Sivaprasad et al., 2019), contrary to the western world (Green et al., 2017). The deficiency status is affected by the host factors (Lachner et al., 2012) such as age, various personal habits (vegetarianism, veganism, alcoholism and smoking), altered physiological states (gastric atrophy and/or pernicious anemia) and therapeutic interventions (medications like metformin, proton inhibitor, or H_2 antagonist). Accordingly, the routine consumption of this vitamin is necessary to prevent any preclinical or clinical symptoms.

The people with plant-based dietary habits are more prone to B12 deficiency (Xie et al., 2021). Fermentation with food-grade organisms and chemical fortification remain the only two ways of incorporating this vitamin to plant-based foods (Bhushan et al., 2019; Xie et al., 2021). The B12 production potential is however, not common among all food-fermenting organisms, and only some strains of propionic and lactic acid bacteria (LAB) have shown potential for food B12 biofortification (Chamlagain et al., 2015; Bhushan et al., 2017; Deptula et al., 2017; Xie et al., 2021). Though, some *Propionibacterium* strains are superior in displaying higher levels of B12 biofortification (Signorini et al., 2018; Xie et al., 2019; Xie et al., 2021), the intracellular nature of B12 biosynthesis is considered as a challenge for B12 release during food-processing and/or digestion (Chamlagain et al., 2021). Moreover,

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https://doi.org/10.1016/j.crfs.2021.09.003
Received 25 May 2021; Received in revised form 6 September 2021; Accepted 9 September 2021
Available online 14 September 2021

Table 1
PCR-related information for amplification of *cbiK* gene and 16S–23S *rRNA*/spacer region.

Sample	Targeted region/(Genus/Species identified)	Primer Sequences	Product size (bp)	Annealing Temperature (°C)	Reference
Genomic DNA	16S–23S *rRNA* intergenic spacer region and its flanking 23S *rRNA*/*Lactobacillus reuteri*	F: 5′-CAGACAATCTTTGATTGTTTAG-3′ R: 5′- GCTTGTTGGTTTGGGCTCTTC-3′	303	60	Song et al. (2000)
Genomic DNA	*16S–23S* spacer/*Lactobacillus rhamnosus*	F: 5′-CAGACTGAAAGTCTGACGG-3′ R: 5′-GCGATGCGAATTTCTATTATT-3′	113	58	Walter et al. (2000)
Genomic DNA	*cbiK* gene	F: 5′-CGGCTATCCCATTCTCCTT-3′ R: 5′-GGCTGCGTTTCCAGATTAT-3′	596	59	Bhushan et al. (2016)

bp, base pairs; *°C*, degree celsius; *DNA*, deoxyribonucleic acid; *F*, forward; *R*, reverse.

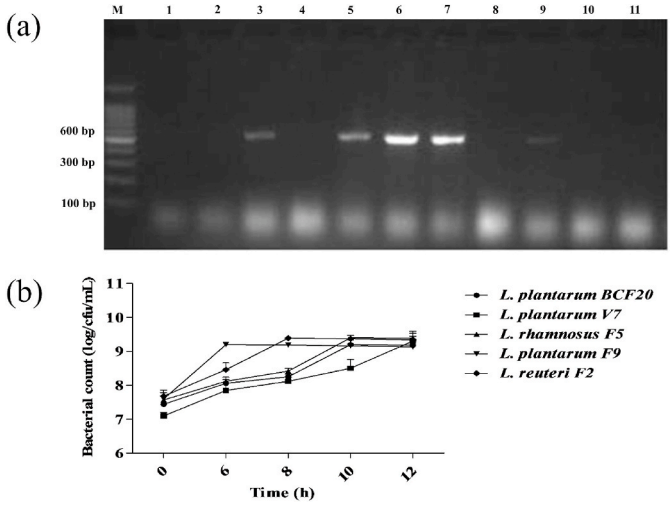

Fig. 1. Selective screening for *cbiK* gene detection in fecal DNA samples (A) and growth curves of selected lactobacilli in B12 free commercial medium (BCM) (B). *cbiK*-gene specific PCR product from tested *Lactobacillus* strains [1, F1; 2, F3; 3, BCF20 (positive control); 4, F6; 5, F2; 6, V7; 7, F5; 8, F4; 9, F9; 10, F7; 11, NCDC745 (negative control). M, Marker DNA.

the claims for probiotic and generally regarded as safe (GRAS) status are limited only to one *Propionibacterium* spp. (*P. freudenreichii*), thereby representing this group less conventional for delivering health benefits *in vivo* (Zarate, 2012). Alternatively, two reports (Masuda et al., 2002; Li et al., 2017) have claimed extracellular B12 production by lactobacilli, which are GRAS organisms of choice for expression of their functionalities *in vitro*, *in situ* and *in vivo*. Hence, a huge scope has arisen to identify potent extracellular B12 producing (or B12 secreting) lactobacilli candidates for future food-fortification and human supplementation strategies.

In a previous report from our lab, the lactobacilli were first isolated from biological samples of human origin, and subsequently screened via a novel *cbiK* gene detection strategy for selection of B12 producing lactobacilli (Bhushan et al., 2016). The *cbiK* gene codes for the enzyme cobalt chelatase that incorporates, anaerobically and in a B12-specific manner, the cobalt ion into the center of the tetrapyrrole ring of cobalamin (Bhushan et al., 2016). In current work, a scope of modification in previous PCR-based methodology was evinced to directly select the human fecal samples with *cbiK* gene positivity, in order to avoid the cumbersome processing of all the samples.

Besides, the selection of appropriate food matrices is also required for development of B12 biofortified functional food products (Bhushan et al., 2020). Soymilk is an aqueous extract of soybeans, a cheaper alternative to cow-milk, and basically is known for its high content of protein, essential amino acids, folate and polyunsaturated/saturated fatty acids (1/1:0.3). It is free from cholesterol, gluten and lactose (Singh et al., 2016; Bhushan et al., 2020; Singh Narayan et al., 2021). Additionally, the absence of B12 makes it a first-choice food-base for cobalamin biofortification using food fermentation (Gu et al., 2012; Bhushan et al., 2017). However, the presence of unusual sugar contents (like stachyose and raffinose) in soy milk would probably make LAB fermentation a somewhat onerous task, and may necessitate addition of some alternative growth enhancers with no negative effect on human health.

For a complex biological system like food fermentation, the optimal design of experiments becomes a tedious proposition to ascertain invariability and to improve effectiveness, especially while negotiating with multilevel factors (Surowiec et al., 2017; Bhushan et al., 2021). Besides the traditional and versatile use of one factor at a time (OFAT) approach due to its simplicity (Dhankhar et al., 2019), the use of generalized subset designs (GSD) has been suggested as a powerful tool to customize the experimental combinations (Surowiec et al., 2017). Further, the Adaptive Neuro Fuzzy Inference System (ANFIS), a combination of a learning tool (Artificial Neural Network) and a robust system (Fuzzy Logic), may predict the effectiveness of statistical experimental designing, the maximal production levels of targeted biomolecule and the interrelationships among fermentation variables.

Considering the afore-mentioned gaps in knowledge, the objectives of this study were (a) to screen, innovatively, the genomic potential of human fecal samples and correspondingly isolated lactobacilli for B12 production (b) to characterize the selected lactobacilli into extracellular

Table 2
Extracellular and intracellular vitamin B12 production levels (μg/L) in lactobacilli fermented samples of B12-free commercial medium (BCM).

Tested strains	*Lactobacillus* species	B12 quantified by UFLC (in μg/L)		B12 quantified by Microassay (in μg/L)	
		Intracellular	Extracellular	Intracellular	Extracellular
Positive std. BCF20	*L. plantarum*	$^{a}24.1 \pm 0.7$	nd	$^{a}97.5 \pm 4.0$	nd
Negative std. NCDC745	*L. plantarum*	nd	nd	nd	nd
F9	*L. plantarum*	$^{b}12.9 \pm 1.5$	nd	$^{b}56.6 \pm 3.2$	nd
V7	*L. plantarum*	$^{c}7.6 \pm 0.3$	$^{a}41.3 \pm 1.6$	$^{c}31.9 \pm 1.9$	$^{a}158.5 \pm 2.2$
F5	*L. rhamnosus*	$^{d}3.1 \pm 0.2$	$^{b}89.3 \pm 2.7$	$^{d}12.9 \pm 1.2$	$^{b}349.6 \pm 3.2$
F2	*L. reuteri*	$^{d}2.7 \pm 0.3$	$^{c}132.2 \pm 1.9$	$^{d}12.8 \pm 2.0$	$^{c}524.9 \pm 6.9$

$^{a, b, c, d}$Values with different letters differ significantly ($p<0.05$ to $p<0.001$). Graphpad-Prism (version 5) was used to analyze the data with 1-way ANOVA using Tukey's post-hoc test.

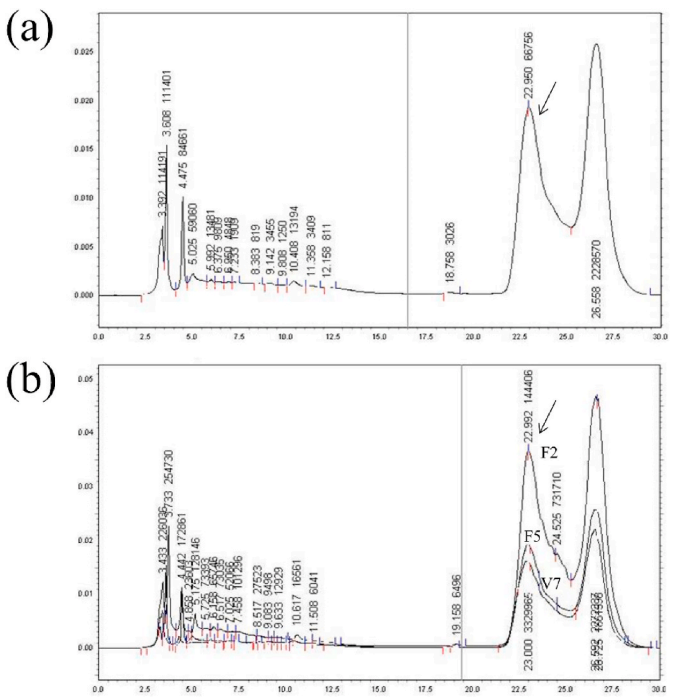

Fig. 2. UFLC overlaid chromatogram showing separation of nutritionally-relevant and biologically-active vitamin B12 (cyanocobalamin) in extracted standard and test samples. Cyanocobalamin peak(s) in panels [A, peak of extracted commercial standard; B, Peaks generated from extracellular cell extracts of *Lactobacillus* strains F2, F5 and V7].

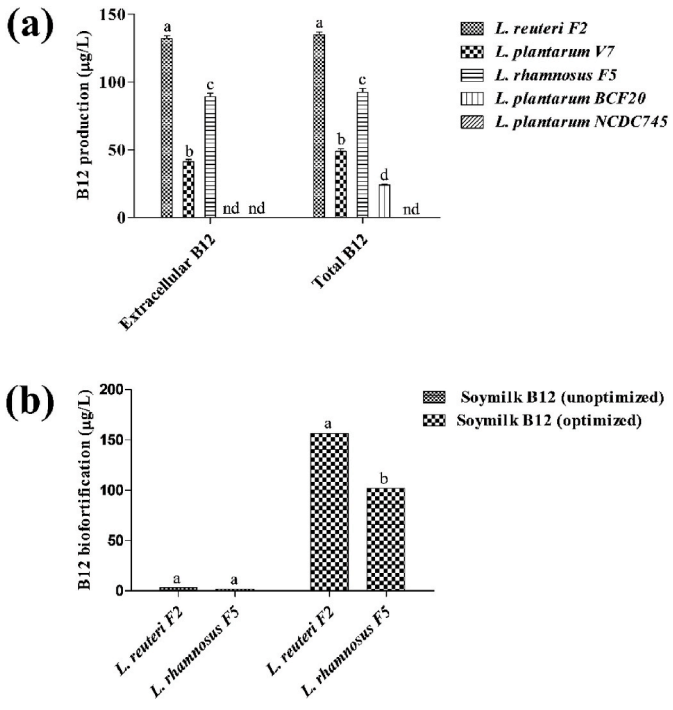

Fig. 3. Comparative bar charts displaying B12-concentrations in extracted samples of lactobacilli fermented BCM [(Extracellular B12), (Total B12)] and soymilk in figure panels (a) and (b), respectively. Data was analyzed with Graphpad-Prism software (version 5.0) for 2-way ANOVA using Bonferroni's test. Different letters on the top of the bars represent the highly significant differences among values ($p<0.01$). *BCM*, B12-free Commercial Medium; *nd*, not detected.

and/or intracellular B12 producing biotypes in B12-free growth conditions (c) to exploit extracellular B12 producing lactobacilli for maximal *in situ* B12 biofortification of soymilk using traditional and advanced experimental-designing tools (OFAT followed by GSD) coupled with evolutionary hybrid statistical approach (ANFIS), with a statistical prediction of final B12 levels and inter-variable interactions during fermentation.

2. Materials and methods

2.1. Chemicals, growth media and bacterial strains

Chemicals, enzymes and growth media were from Sigma-Aldrich (St. Louis, USA), unless specified separately. All PCR-related ingredients were from Thermo Fisher Scientific (Waltham, USA). The reference strains for B12 production, *L. plantarum* BCF20 (positive standard), *L. plantarum* NCDC745 (negative standard) and *L. delbrueckii* subsp. *lactis* ATCC7830 (auxotrophic mutant), and 62 other lactobacilli (Table S1) were obtained from National Collection of Dairy Cultures (NCDC, NDRI, Karnal, India). The de-Man, Rogosa and Sharpe (MRS) broth (HIMEDIA, Mumbai, India) was used for normal reviving and activation of cultures, while B12-free commercial medium (BCM) was for B12 production. Anaerobic and microaerophilic conditions, using respective Anaerocult gas packs (Merck KGaA, Darmstadt, Germany), were provided as per requirements. The cultures were maintained in glycerol (30%, *v/v*) at −20 °C.

2.2. PCR-based B12 screening of human fecal samples and correspondingly recovered lactobacilli

Infant fecal samples (n = 15) were collected from baby diapers provided by corresponding mothers. Sample swabs in MRS broth tubes (pH = 5.0) were incubated at 37 °C for 14–16 h in complete anaerobiosis (Anaerocult-A, Merck) for selective enrichment of fecal lactobacilli. The fecal samples and bacterial strains were subjected, separately, to DNA isolation using adapted protocol (Pospeich and Newmann, 1995). PCR amplification (Bio-Rad, Hercules, CA, USA) of *cbiK* gene was performed using primers (Table 1) designed previously in our lab (Bhushan et al., 2016). The genomic DNA from strains BCF20 and NCDC745 were used as positive and negative controls, respectively, for PCR reaction. The bacillary isolates recovered from *cbiK* positive fecal samples were also tested for presence of *cbiK* gene and later characterized on species level using species-specific PCR (Table 1).

2.3. Vitamin B12 bioproduction (extracellular and/or intracellular) in BCM

2.3.1. Inoculum and fermentation

All selected lactobacilli isolates were repeatedly sub-cultured (10 times) in BCM to remove carryover B12 and to activate tested culture for final B12 fermentation in the said medium. The B12 production (extracellular and/or intracellular) was achieved via a sequential two-phase fermentation strategy (i.e., anaerobic followed by micro-aerophilic phase) and the incubation time for first anaerobic phase was determined on the basis of growth curves of tested strains (Bhushan et al., 2016). The time (4 h) for microaerophilic incubation was kept constant. The isolates with superior extracellular B12 production potential were characterized as B12-secreting biotypes and subjected to further experimentation.

2.3.2. Vitamin B12 analysis in fermented BCM samples

2.3.2.1. Extraction. The two types of culture extracts (CE), CE (I) for intracellular and CE (E) for extracellular, were generated from cell pellet and culture supernatant, respectively. The B12 extraction protocol

Table 3
Vitamin B12 levels (μg/L) in lactobacilli fermented soymilk after OFAT optimization of three variables (inoculum level, glucose & fructose concentration).

Variables	Vitamin B12 concentration (μg/L) as per tested methodology and represented as (Mean ± SD)							
	HPLC	Micro assay	HPLC	Micro assay	HPLC	Micro assay	HPLC	Micro assay
Inoculum level (%)	1		3		5		10	
L. reuteri F2	2.8 ± 0.3	9.4 ± 1.3	8.1 ± 1.0	37.3 ± 7.4	[a]**14.9 ± 1.7**	59.2 ± 5.7	9.2 ± 0.5	38.7 ± 5.3
*L. plantarum*V7	0.7 ± 0.1	2.9 ± 1.0	2.7 ± 0.4	12.9 ± 3.2	[b]**4.4 ± 0.7**	17.7 ± 2.7	3.1 ± 0.0	13.6 ± 2.9
*L. rhamnosus*F5	1.8 ± 0.3	7.3 ± 1.2	[b]**5.2 ± 0.5**	21.6 ± 3.3	3.8 ± 0.2	15.27 ± 1.7	3.7 ± 0.3	14.3 ± 2.3
Glucose conc. (%)	2		4		6		8	
L. reuteri F2	18.3 ± 1.2	82.3 ± 5.2	[a]**25.8 ± 2.2**	126.9 ± 12.2	23.9 ± 1.4	106.7 ± 9.5	19.7 ± 0.9	81.3 ± 9.1
*L. plantarum*V7	5.3 ± 0.6	22.4 ± 2.1	[b]**7.3 ± 1.2**	33.6 ± 4.2	5.4 ± 0.5	22.4 ± 2.1	5.0 ± 0.8	21.3 ± 1.5
*L. rhamnosus*F5	6.9 ± 0.9	27.8 ± 2.9	9.3 ± 3.0	39.9 ± 1.2	[c]**15.6 ± 2.0**	67.9 ± 2.9	10.6 ± 0.9	42.6 ± 3.3
Fructose Conc. (%)	1		2		3		4	
L. reuteri F2	93.2 ± 2.5	369.2 ± 14.6	[a]**145.6 ± 3.5**	601.7 ± 10.9	108.1 ± 2.9	428.5 ± 13.9	79.3 ± 2.1	327.5 ± 15.5
*L. plantarum*V7	33.2 ± 2.8	128.6 ± 2.6	[b]**45.2 ± 1.6**	186.3 ± 6.9	36.4 ± 1.9	134.7 ± 16.2	29.9 ± 1.5	121.9 ± 1.6
*L. rhamnosus*F5	75.1 ± 3.1	307.6 ± 8.1	[c]**89.4 ± 1.7**	362.9 ± 13.9	75.9 ± 3.8	319.4 ± 15.8	69.9 ± 1.2	278.9 ± 14.2

[a, b, c]Values with different letters differ significantly ($p<0.05$ to $p<0.001$). Graphpad-Prism (version 5) was used to analyze the data with 1-way ANOVA using Tukey's posthoc test.
Data in bold numeric is showing the maximum production levels with either of standardized variable.

(Taranto et al., 2003; Bhushan et al., 2016) included physical disruption on a shaker (glass beads, 0.1 mm) and boiling in extraction buffer (0.1 M Na2HPO4, pH 4.5 with 0.005% KCN), to obtain a more stable cyanocobalamin form. The commercial standard of cobalamin was added in BCM and extracted in the same manner. The samples of cyanocobalamin were filtered through 0.22 μm filters (Pall biopharmaceuticals, USA).

2.3.2.2. Purification. The extracted samples were purified as per adapted protocol (Taranto et al., 2003) with modifications, using pre-activated C18 solid-phase extraction (SPE) cartridges with sequential steps of loading, elution, re-loading and re-elution. The eluents were collected in amber colored tubes, vacuum dried and dissolved in 500 μL of milli-Q water, and filtered through 0.22 μm membranes and stored at −20 °C.

2.3.2.3. UFLC for precise detection and quantification. The CEs were separated in dark using an ultra-fast liquid chromatography (UFLC) system (Shimadzu, Japan) as per lab protocol (Bhushan et al., 2016, 2017). Briefly, a reverse phase C18 column (Phenomenex, Luna 5μ C18 (2) 100 A, 250 × 4.6 mm) was used with elution gradient of acetonitrile and water for UV detection of cyanocobalamin at 360 nm. The lactobacilli with highest extracellular B12 production potential were selected for soymilk fermentation experiments.

2.4. Microassay for detection, quantification and bioavailability

The phenotypic production, quantification and bioavailability of bacterial B12 in CEs was also confirmed by an AOAC approved gold standard bioassay as tested previously (Bhushan et al., 2016), using *L. delbrueckii* subsp. *lactis* (*L. leichmannii*) ATCC7830 as a B12 auxotroph, and *L. plantarum* BCF20 as a positive control. The growth of mutant was determined by measuring optical density (OD) at 595 nm after 20 h of incubation at 37 °C. For B12 quantification, a standard growth curve of mutant was obtained with predefined concentrations of extracted cyanocobalamin standard, and an equation was extrapolated.

2.5. Vitamin B12 biofortification of soymilk via lactobacilli fermentation

2.5.1. Preparation of soymilk and fermentation conditions

In order to prepare the soymilk, 100 g of soybeans (*Glycine max*) were soaked overnight with 500 mL water containing 0.5% $NaHCO_3$. The washed soybeans were heated, dehulled, grinded and blended 4x with water using the blender (Philips HL 250W Hand Blender, India) to obtain the filtered soymilk, which was heated at 90 °C for 10 min before use.

The selected B12-secreting *Lactobacillus* isolates were exploited for B12 fermentation in soymilk. A previously standardized two-phase growth (i.e. anaerobic followed by microaerophilic) strategy was applied (Bhushan et al., 2017) to obtain initial B12 levels. The first anaerobic incubation (10 h) was required for log phase growth and synthesis of B12 intermediates (or cobamides), while the second microaerophilic incubation (10 h) was necessitated for synthesis and/or replacement of lower ligand DMB to complete the structural organization of active B12 molecule (Deptula et al., 2017). Briefly, the freshly prepared washed bacterial inoculum was initially tested for growth in 15 mL of soymilk, and finally inoculated in amber colored screw-capped bottles (100 mL) for two-phase fermentation at 37 °C for 20 h, with an initial cell concentration of 7–8 log cfu/mL. The care was taken to carry out fermentation and extraction procedures under minimal exposure to light.

2.5.2. Optimization strategies: OFAT → GSD → ANFIS

To attain the highest B12 biofortification levels, the traditional (OFAT) and advanced (GSD) experiment-designing tools coupled with evolutionary-hybrid (ANFIS) algorithms were pursued.

2.5.2.1. OFAT approach. The OFAT strategy was tested on bacterial inoculum levels (1%, 3%, 5%, and 10%) and external growth-promoting additives [glucose (0%, 2%, 4%, 6%, and 8%) and fructose (0%, 1%, 2%, 3% and 4%)]. The nutrient addition of glucose and fructose was adapted from a previous report (Gu et al., 2015), but with modifications in percentages.

2.5.2.2. GSD-ANFIS approach. The OFAT optimized parameters were followed during GSD-ANFIS optimization. Generalized Subset Designs tool (MODDE 12.0 software, Sweden) was employed to construct an experimental design with highly justifiable number of fermentation runs, although working on a high number of variables; temperature (30, 34, 37 and 40 °C), time (12, 16 and 20 h), and pH (6.0, 7.0 and 8.0). The experiments were designed and performed in triplicates for selected isolates. For ANFIS, the fuzzy logic toolbox of MATLAB 17.0 was used. The input variables were preconditioned and combined linearly by using the rules of Takagi-Sugeno (TS). The TS fuzzy rules were described by mapping input-output variables in terms of IF-THEN rule (Appendix S1). The data obtained from GSD-based experimental runs was arranged into three groups named training, testing and validation. In order to avail the modeling network of the actual fitting, a 4 × 36 inputs, namely temperature (30–40 °C), time (12–20 h) and pH (6–8), corresponding to 36 values of 1 output (B12 concentration, μg/L) were used. The results were represented by 3-D contour plots, and the inter-variable interactions for enhanced production of B12 in fermented soymilk were studied.

Table 4
GSD experimental design matrix and corresponding obtained B12 fortification levels (real values of experimental runs and predicted values generated using ANFIS) observed in fermented soymilk for tested variables (temperature, time and pH). The planned 36 experiments generated 108 values when performed in triplicates. Hereby, the 3 values for each experimental run have been arranged and feed to MATLAB software so as to obtain the best-fit ANFIS model and appropriate predictions with lowest root mean square values and highest R value (approaching near to 1.0).

No.	Tested conditions	B12 biofortification (μg/L) with *L. reuteri* F2		B12 biofortification (μg/L) with *L. rhamnosus F5* (Mean ± SD)	
		Experimental (Mean ± SD)	ANFIS predicted	Experimental (Mean ± SD)	ANFIS predicted
1	30 °C; 12h; 6	75.9 ± 1.7	75.9	48.9 ± 2.4	48.9
2	30 °C; 12h; 7	94.8 ± 4.3	94.8	56.5 ± 2.2	56.5
3	30 °C; 12h; 8	64.3 ± 4.2	64.3	35.1 ± 4.4	35.1
4	30 °C; 16h; 6	84.9 ± 2.0	84.9	55.4 ± 2.0	55.4
5	30 °C; 16h; 7	104.5 ± 2.9	102.9	64.2 ± 2.9	62.7
6	30 °C; 16h; 8	70.7 ± 5.4	70.4	41.6 ± 2.1	42.2
7	30 °C; 20h; 6	84.8 ± 2.6	84.8	56.4 ± 1.7	56.4
8	30 °C; 20h; 7	100.6 ± 3.4	103.6	63.1 ± 3.5	63.8
9	30 °C; 20h; 8	67.5 ± 1.7	68.4	40.6 ± 2.9	38.9
10	34 °C; 12h; 6	97.2 ± 3.5	97.2	57.3 ± 2.0	57.3
11	34 °C; 12h; 7	120.4 ± 4.5	120.5	63.0 ± 4.2	63.0
12	34 °C; 12h; 8	76.9 ± 2.9	76.9	44.4 ± 4.5	44.4
13	34 °C; 16h; 6	103.6 ± 3.6	103.7	66.2 ± 1.9	66.2
14	34 °C; 16h; 7	126.5 ± 2.9	126.5	70.4 ± 3.5	70.4
15	34 °C; 16h; 8	77.0 ± 1.4	77.0	48.9 ± 4.4	48.9
16	34 °C; 20h; 6	99.7 ± 2.2	98.5	66.0 ± 2.9	67.7
17	34 °C; 20h; 7	122.1 ± 3.9	122.1	69.2 ± 3.8	69.2
18	34 °C; 20h; 8	70.8 ± 2.0	70.8	47.8 ± 1.7	47.8
19	37 °C; 12h; 6	136.4 ± 2.8	136.5	68.4 ± 2.2	68.4
20	37 °C; 12h; 7	156.2 ± 3.6	156.3	76.4 ± 3.5	76.4
21	37 °C; 12h; 8	94.7 ± 2.5	94.7	50.5 ± 3.9	50.5
22	37 °C; 16h; 6	133.7 ± 1.1	133.9	80.9 ± 3.8	81.9
23	37 °C; 16h; 7	154.9 ± 4.2	154.9	101.7 ± 3.4	101.7
24	37 °C; 16h; 8	91.1 ± 0.9	92.2	56.6 ± 3.3	56.3
25	37 °C; 20h; 6	127.5 ± 2.6	126.7	79.7 ± 4.5	81.5
26	37 °C; 20h; 7	147.5 ± 4.2	145.2	96.6 ± 2.9	94.9
27	37 °C; 20h; 8	88.2 ± 2.8	86.9	50.0 ± 2.7	48.5
28	40 °C; 12h; 6	129.1 ± 4.8	129.1	67.5 ± 4.0	67.5
29	40 °C; 12h; 7	151.3 ± 4.6	151.3	75.1 ± 4.5	75.1
30	40 °C; 12h; 8	104.4 ± 1.9	104.8	47.3 ± 2.9	48.9
31	40 °C; 16h; 6	125.9 ± 1.7	125.9	80.8 ± 2.9	80.8
32	40 °C; 16h; 7	147.3 ± 2.1	147.3	95.9 ± 2.9	95.9
33	40 °C; 16h; 8	107.9 ± 3.0	109.1	52.2 ± 3.5	53.3
34	40 °C; 20h; 6	117.3 ± 2.1	117.2	78.2 ± 2.8	77.1
35	40 °C; 20h; 7	145.3 ± 2.5	145.3	94.2 ± 2.9	94.2
36	40 °C; 20h; 8	103.4 ± 2.4	102.6	50.3 ± 2.5	48.9

°*C*, degree Celsius; *h*, hour; μg/L, microgram per liter; *SD*, standard deviation; maximum production highlighted in bold.

2.6. Vitamin B12 analysis in fermented soymilk samples

B12 extraction from fermented soymilk was somewhat different from what has been described earlier for the culture broth. The soy-curd sample (10 g) was mixed with 90 ml of 50 mM sodium acetate buffer (pH 4.0 adjusted with glacial acetic acid), 1 mL potassium cyanide (0.5%), 0.5 g of amylase and 2 g of pepsin and incubated at 37 °C for 1 h with mixing at an interval as suggested elsewhere (Marley et al., 2009). The aqueous-mix was then autoclaved at 121 °C for 20 min. The obtained supernatant was purified through solid phase extraction cartridges and eluent was tested with microbiological assay, for detection and bioavailability, and with UFLC, for precise quantification of produced B12 in a similar manner as described for BCM.

2.7. Statistical analysis

The values generated from individual experiments were analyzed for estimation of errors and expressed in Mean ± SE. Besides use of Microsoft Office (2017) for tabulation and descriptive representation of data, GraphPad Prism (version 5) was used for Analysis of Variance (ANOVA) among values of statistical significance. The fuzzy logic toolbox of MATLAB 17.0 was used for prediction and validation related ANFIS work. MODDE 12.0 software (MKS Data Analytics Solutions, Umeå, Sweden) was used to generate all GSD based experimental designs in this study.

3. Results and discussion

3.1. PCR-based B12 screening of human fecal samples and correspondingly recovered lactobacilli

The 4 infants' fecal samples and correspondingly recovered lactobacilli were found positive for *cbiK* gene (Fig. 1A), and the isolates showed repetitive growth in BCM (Fig. 1B). In the present study, it was intended to modulate the concentration of B12 producing lactobacilli through modified growth conditions (acidification and complete anaerobiosis of medium), and to detect *cbiK* gene directly in fecal samples as pre-isolation steps. This approach reduced the time and efforts required for processing, isolation, purification and maintenance of cultures recovered from all the samples.

All bacillary, Gram-positive and catalase-negative isolates, derived from positive fecal samples after selective enrichment, were confirmed to be lactobacilli through genus-specific PCR reaction. Moreover, 2 of 4 *cbiK*-positive isolates (V7, and F9) were characterized through species-specific PCR as *Lactobacillus plantarum*, 3rd strain (F2) as *L. reuteri* and 4th one (F5) as *L. rhamnosus*. In terms of novelty, this is the first report claiming for *L. rhamnosus* species as vitamin B12 producer, and for *cbiK* gene in its genomic DNA (Fig. 1A). However, a few strains among other

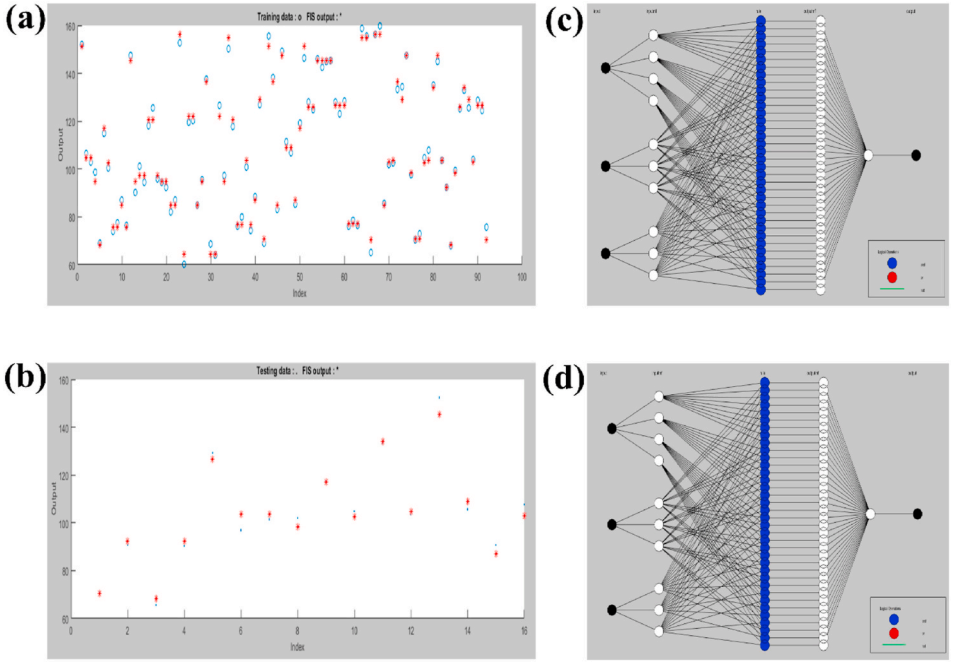

Fig. 4. The representative FIS plots for ANFIS training [panel (a)] and testing performance [panel (b)], along with structural plots for *L. reuteri* F2 [panel (c)] and *L. rhamnosus* F5 [panel (d)] are shown in this figure.

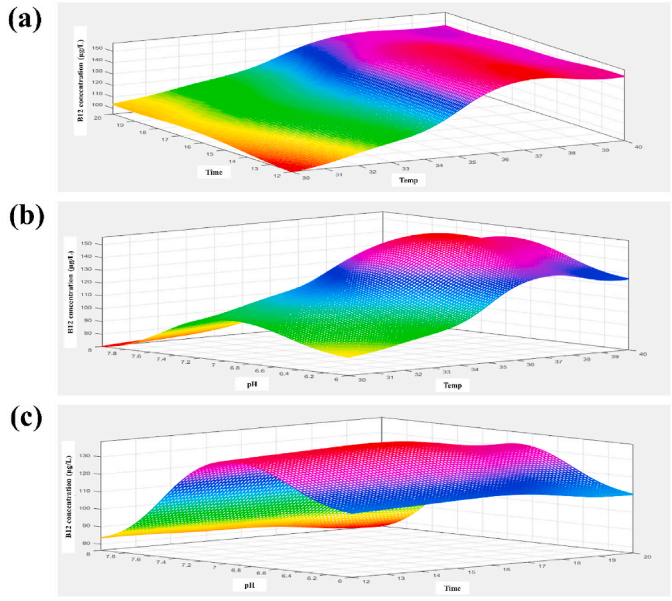

Fig. 5. The 3D contour plots describing inter-variable relationships during optimized soymilk B12 biofortification process for best strain *L. reuteri* F2 are displayed for tested variables; temperature and time [panel (a)], temperature and pH [panel (b)], and time and pH [panel (c)].

Lactobacillus species, like *L. reuteri* (Taranto et al., 2003), *L. plantarum* (Masuda et al., 2012; Bhushan et al., 2016, 2017), *L. rossiae* (De Angelis et al., 2014), and *L. coryniformis* (Masuda et al., 2012) are already known for this functionality. The researchers **suggested horizontal** gene transfer as the responsible mechanism for acquiring these genes (*cob-pdu* cluster) by lactobacilli from their respective ecological niches (Taranto et al., 2003; Masuda et al., 2012; Bhushan et al., 2016).

Surprisingly, all of NCDC lactobacilli (no. 62) were found negative for the presence of *cbiK* gene in their genomic DNA (Fig. S1), hence not subjected to further characterization. B12 production has always been reckoned as a rare and strain-specific functionality among lactobacilli (LeBlanc et al., 2013; Bhushan et al., 2016).

3.2. Vitamin B12 bioproduction (extracellular and/or intracellular) in BCM

3.2.1. Inoculum and fermentation

The B12 production was carried out in B12-free growth conditions, in BCM, by selected isolates of choice. The inoculum preparation through repetitive subculturing was found effective in removing the carried-over B12 from MRS and in sensitizing the strains for B12 production in cobalamin-free growth conditions (Bhushan et al., 2016), that is evident through their fast growth in BCM (Fig. 1B). On the basis of their growth curves, the strains (F9, F2, BCF20, F5 & V7) attained late log-phase in time intervals of 6, 8, 10, 10 & 12–14 h, respectively, when activated mid-log phase cultures were taken as inoculum at 1%.

3.2.2. Quantitative comparison of the UFLC results with microassay

The extracted culture extracts, CE (I) & CE (E) were purified through SPE cartridges and were tested for quantification of cyanocobalamin through UFLC and microassay. The values enlisted in Table 2 are compared for evaluating the specificity of protocols used. Two of the tested strains (*L. plantarum* BCF20 & F9) were found to be negative for extracellular B12 production with both the techniques, while isolate namely *L. reuteri* F2 and *L. rhamnosus* F5 displayed this functionality and may be designated as B12-secreting organisms. The UFLC analysis confirmed the B12 production [in either of CE (I and E)] in *cbiK*-positive lactobacilli but not in *cbiK*-negative strain NCDC745, hence, the results validated the preciseness of PCR-based screening strategy previously developed in our lab (Bhushan et al., 2016). The present study reports for an overestimation of the B12 content through microbiological assay (Table 2), thereby confirmed the likely presence of inactive corrinoids in the fermented BCM, as also reported previously for propionibacteria (Chamlagain et al., 2015, 2017) and lactobacilli (Taranto et al., 2003). The auxotrophic mutant is likely to consume all corrinoids present in tested cell extracts along with the active B12 (Taranto et al., 2003; Chamlagain et al., 2015; Bhushan et al., 2016). Hence, we used a sophisticated analytical UFLC for characterization (inactive or active-B12)

and precise quantification of extracted and purified B12, as suggested by researchers worldwide (Quesada-Chanto et al., 1998; Taranto et al., 2003; Chamlagain et al., 2015, 2017; Bhushan et al., 2016, 2017; Edelmann et al., 2016; Deptula et al., 2017; Bao et al., 2019; Qiu et al., 2019) and by AOAC First Action *Official Method*^{SM} 2014.02 (Giménez and Martin, 2018). UFLC quantified the cyanocobalamin on the basis of similar retention times and peak patterns for the standard and the test samples (Fig. 2). Moreover, the cyanocobalamin spiking elevated only the peak of choice (data not shown), thereby confirming the nutritionally-relevant nature of B12 produced by the selected isolates. However, the definitive use of mass spectrometry for elemental/electronic characterization of produced B12 is suggested (Chamlagain et al., 2015, 2017).

In terms of intracellular B12 level, the production in reference strain BCF20 (24.1 ± 0.7 μg/L) was in accordance with the values reported for the same strain previously (Bhushan et al., 2016), but significantly higher ($p<0.05$) than other tested isolates (2.7 ± 0.3 to 12.9 ± 1.5 μg/L) in current work. In accordance with a previous report (Bhushan et al., 2016) the reference *L. plantarum* BCF20 strain was found positive only for intracellular B12 production. Keeping in mind the light-sensitive nature of B12, the intracellular B12 production during fermentation may also be an equally important strategy for food biofortification.

In terms of the extracellular production of B12 (Fig. 3a & Table 2), 3 of 4 isolates (*L. reuteri* F2, *L. plantarum* V7 and *L. rhamnosus* F5) showed production in CE (E) in a range of 41.3 ± 1.6 to 132.2 ± 1.9 μg/L, which is higher than 60–98 μg/L as reported by Li et al. (2017). The pioneering report for extracellular B12 production in LAB, although quite low (2 μg/L), was for *L. plantarum* strain isolated from Japanese pickle Nukazuke (Masuda et al., 2002). In comparison, *L. reuteri* F2 in the present study, secreted remarkably higher (61-fold) B12 in BCM (132.2 ± 1.9 μg/L) with a significant difference of $p<0.001$. It is noteworthy that B12 secretion isolates investigated in this study corresponded to 84–97% of the total cobalamin production (Fig. 3a), which is significantly higher ($p<0.001$) than 8.9–9.6% reported by (Li et al., 2017). We report the highest ever ratio (0.97:1.00) among extracellular-vs total-B12 with *L. reuteri* strain F2 in BCM, thereby, confirming the prolific B12 secreting phenotype of this strain. Unsurprisingly, *L. plantarum* NCDC745, a *cbiK*-negative strain, couldn't produce B12 in either of the samples (Fig. 3).

The scarcity in reports for extracellular B12 production among GRAS organisms encouraged us to find novel indigenous strains and optimize their vitamin production. Many food-grade organisms, including *Propionibacterium* and *Lactobacillus* species, have been reported for intracellular B12 production (Taranto et al., 2003; Chamlagain et al., 2015, 2017, 2021; De Angelis et al., 2014; Bhushan et al., 2016, 2017; Deptula et al., 2017), hence the current work on extracellular B12 production remains pertinent.

3.2.3. Importance of microbiological assay

The microassay has been approved by Official methods of analysis of AOAC International as a gold standard method not only for the phenotypic confirmation of B12 bio-production but also for the vitamin bioavailability and sensitivity (Chamlagain et al., 2015; Bhushan et al., 2016). In the present study, the CE (I) of all tested lactobacilli supplemented the growth of auxotrophic mutant ATCC7830, confirming both detection and bioavailability of intracellular B12 in a living system (Bhushan et al., 2016). Also, the mutant showed high sensitivity towards changing concentrations of the commercial B12-standard, as displayed through a linear standard growth curve (Fig. S2) with high R (>0.99) value. A few reports (Santos et al., 2011; De Angelis et al., 2014) have used microbiological assay as a sole method for estimation of bacterial B12, probably because of its low-cost. But, the indispensable nature of this assay is recognized in the current work for phenotypic confirmation and bioavailability of bacterial B12 produced in spent culture extracts, and not for quantification of active B12.

3.3. Vitamin B12 biofortification of soymilk via lactobacilli fermentation

The global presence of B12 deficiency and its irreversible health abnormalities in aggravated health conditions compelled researchers all over the world to develop some novel B12 fortification strategies using indigenous LAB (LeBlanc et al., 2013). In line, the selected B12 producing lactobacilli (*L. reuteri* F2, *L. plantarum* V7 & *L. rhamnosus* F5) were isolated from fecal samples of indigenous population and exploited for fermentation-based B12 biofortification of soymilk. Interestingly, the 3 selected lactobacilli showed potential for soymilk curdling after 4–6 h of fermentation, with appropriate relationship of pH and acidity (data not shown). The exogenous addition of sugars might be the reason behind fast curdling of soymilk, as also suggested elsewhere (Gu et al., 2015). The detection and precise quantification of cyanocobalamin in fermented soymilk samples showed a B12 biofortification potential of both of the tested isolates (Fig. 3b).

3.3.1. Optimization strategies: OFAT → GSD → ANFIS

The OFAT-based optimization of variables (inoculum, glucose and fructose) significantly ($p<0.05$) enhanced the B12 biofortification levels (Table 3). The highest production level (>145 μg/L), obtained after sequential optimization of these 3 parameters in a two-phase fermentation by *L. reuteri* F2, was significantly higher ($p<0.01$) than the initial conditions. Interestingly, even with a lower inoculum level (3% versus 5% for F2 & V7), *L. rhamnosus* F5 produced 15% more B12 in comparison to *L. plantarum* V7, thereby showing more growth and cobalamin-production potential of F5 in soymilk. Yet, *L. reuteri* strain F2 outcompeted other two strains in all other aspects (Table 3). The researchers have suggested the oligosaccharide breakdown and proteolytic activities as growth promoters for soymilk-fermenting lactobacilli (Singh et al., 2020), however, that might not be completely fitting into our fermentation model due to exogenous addition of simple sugars. The addition of fructose beyond a level (i.e., 2%) resulted invariably in a decline of final B12 bioproduction in all tested lactobacilli (Table 3), as reported by others (Gu et al., 2015).

The GSD, an advanced experimental-designing technique, reduced the experimental runs to 36, while accounting, simultaneously, for all of the possible permutations and combinations. The highest B12 biofortification values (156.3 ± 3.6 μg/L) generated after suggested experimental runs have been listed in Table 4. In line with a previous study (Surowiec et al., 2017), our results demonstrated that GSD-based selection of experimental design subsets was appropriate and cost-effective.

The ANFIS is a statistical approach for data handling, which gets its origin from biological neurons and the gene theory of evolution, providing an efficient convergence criterion of hiding nodes that results in fitting of a realistic function. The prediction model in current study was highly authentic and best fit, with the appreciable RMSE (2.5258 and 2.4718) and R values of 0.9995 and 0.9986 for *L. reuteri* F2 and *L. rhamnosus* F5, respectively, as suggested by others (Dhankhar et al., 2019). The ANFIS training and testing performance FIS plots and structure plots can be seen in Fig. 4. Other researchers have also reported ANFIS prediction alone (or in combination with GSD designing) as accurate tool for optimization, prediction and validation of biological processes with high R values of 0.957 (Pérez et al., 2018), 0.99 (Dhankhar et al., 2019) and 0.9951 (Karri et al., 2021). With promising results on bioproduction and sensory (data not shown), the optimized B12 biofortification process may be considered viable on industrial, economic and environmental scales (Pérez et al., 2018). The 3D contour plots showed the inter-variable relationships (Fig. 5). For the best B12 producing strain *L. reuteri* F2, the temperature range of 36–38 °C in comparison to range 30–35 °C, could be considered favorable, as optimum biofortification levels obtained at 37 °C. A sharply decreasing curve was seen for B12 biofortification when soymilk fermentation with best strain *L. reuteri* F2 started at initial alkaline conditions and lasted for a longer period of time (after 17 h). The highest B12 production (156.3

± 3.6 μg/L) at 37 °C could be the desirable condition for *in situ* food fortification and for *in vivo* expression of this functionality, but require display of similar results *in vivo*. *L. rhamnosus* F5 also fortified soymilk, but the yield of 101.7 ± 3.4 μg/L was comparatively ($p<0.01$) lower (Fig. 3b). Both *Lactobacillus* strains were isolated from human sources that may be the reason behind their optimal physiological activity (here in terms of B12 production) at or around 37 °C.

The *in situ* B12 fortification level of 156.3 ± 3.6 μg/L with *L. reuteri* F2 in current work was higher than 141.7 μg/kg with *L. reuteri* strain (Bao et al., 2019), and was somewhat lower than 180 μg/L with *L. reuteri* ZJ03 (Gu et al., 2015). The significantly higher production levels of 357.0 ± 8.0, 742.0 ± 18 and 1230.0 ± 58.0 μg/kg dry weight have been reported for *P. freudenreichii* DSM 20271 (Xie et al., 2019), for *P. freudenreichii* DSM 20271 (Xie et al., 2021), and for co-fermentation of *Rhizopus* spp. and *P. freudenreichii* strain 184 (Signorini et al., 2018). Nevertheless, the appreciable output of the present investigation was the short fermentation time of 12 h, which was multifold lower (2–10 times) than 24–120 h observed with above-mentioned reports on prolific B12 producer *P. freudenreichii*. Moreover, LAB fermentation has been known to reduce the objectionable beany flavor of soymilk, and to minimize the levels of non-digestible oligosaccharides present (Singh et al., 2020). An acceptable sensorial profile, along with no pungent odor, was obtained for the final product (data not shown). In terms of fortification per serving size, the fermented product developed and characterized in the present study provided ~2-fold higher ($p<0.05$) B12 in comparison to others (Edelmann et al., 2016). Notably, one serving size (100 mL) of any of this product may fulfil the human Recommended Dietary Allowance (RDA, 2.4 μg/day) of B12, even if processing and bioavailability losses are considered, e.g. 50% in total.

4. Conclusions

B12 production and its secretion into extracellular matrices are acknowledged to be strain specific attributes. In the current work, the genotypic-screening-protocol for direct selection of target fecal samples and useful bacteria was innovatively and successfully employed. Moreover, the genomic screening has proved its importance and validity after demonstrating the B12 bioproduction potential for the first time in *L. rhamnosus* species. The fermentation abilities of *L. reuteri* and *L. rhamnosus* in commercial medium and soymilk confirmed their versatile and resilient nature towards different growth substrates. The use of such prolific extracellular B12 producing organisms could be a promising alternative to chemical fortification of B12-free plant food. However, the precautions for light-sensitive nature of B12 should always be under consideration. The simple and traditional optimization approach (like OFAT) judiciously facilitated the optimization experiments. It was coupled with advanced designing (GSD) and evolutionary hybrid tools (ANFIS) while mapping the variable relationships and developing a best fit optimization model of B12 fortification. *L. reuteri* fermentation of soymilk is a prospective approach to fulfill the daily human RDA with nutritionally-relevant and biologically-active B12.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval

Neither experimental animals nor human subjects were directly involved in the study. Human fecal samples were collected from baby diapers, provided by corresponding mothers. Hence, no scope arose for ethical clearance from animal/human ethical committees. All microbes used in the study were food-grade and rendered present research away from any compass of bio-safety clearance.

CRediT authorship contribution statement

Manorama Kumari: Investigation, experimental, Writing – original draft, Writing – review & editing. **Bharat Bhushan:** Investigation, technical support, Writing – original draft, Writing – review & editing. **Anusha Kokkiligadda:** Data curation. **Vikas Kumar:** Investigation, MATLAB, Formal analysis. **Pradip Behare:** Investigation, co, Supervision. **S.K. Tomar:** Investigation, Supervision, technical support, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Manorma Kumari acknowledges Indian Council of Agricultural research (ICAR), India for providing her the research scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2021.09.003.

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