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Evaluation of environmental stress tolerance of extracellular vitamin B_{12} producing lactobacilli cultures

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Abstract
We investigated environmental stresses tolerance of the extracellular vitamin B_{12} producing lactobacilli cultures and confirmed the screening strategy of vitamin B_{12} producing *Lactobacillus* strains, isolated from the infants' fecal sample. Three cultures (*L. plantarum* NCDC958 and NCDC957, and *L. rhamnosus* NCDC953) endowed with extracellular vitamin B_{12} producing ability from our previous work were amplified for *cbiK* gene with new primers designed in this study and their characteristics in tolerance to environmental stresses (such as temperature, salt, sugar, and ethanol) were studied for the first time with both optical density (OD) measurements and pour plate count method. We confirmed amplification of the 257 bp *cbiK* gene in these cultures, which codes for important B_{12}-structural enzyme cobalt chelatase and successfully selected B_{12}-producing strains. These cultures showed tolerance upto 6% NaCl, 30% sucrose, 42 °C temperature and 8% ethanol. Importantly, extracellular vitamin B_{12}-producing culture *L. rhamnosus* has first-time been reported for the environmental stress tolerance.

Keywords: *CbiK* gene, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, vitamin B_{12}, environmental stress

Introduction
Vitamin B_{12} is an essential vitamin that functions as a cofactor in fatty acid and amino acid metabolism, hemoglobin synthesis, and energy production as well as DNA synthesis and regulation (Nielsen *et al.*, 2012) [12]. It is present in different food varieties but derived mainly from animal source, such as milk, meat and eggs. Therefore, vegetarians are more likely to be prone to vitamin B_{12} deficiency. Vitamin B_{12} deficiency affects several systems, and consequences vary in severity from slight fatigue to severe neurologic impairment (Langan & Goodbred, 2017) [7]. Hence, this clinically important deficiency can be overcome by vitamin B_{12} bioenriched plant-based food product.

The Lactic Acid Bacteria (LAB) are regarded as generally recognized as safe (GRAS) because of their ubiquitous use in food and their unique role in the healthy microflora of human mucosal surfaces. The genus *Lactobacillus* is member of LAB, displays the dynamic interactions within animal and plant kingdoms and also found in a close association with fermented foods (sauerkraut, pickles, cheese, yogurt, sausage, fish, fish- and soy- sauce, sourdough bread, and animal silage), and beverages (George *et al*., 2018) [5]. The microbial diversity of fermented food improve shelf life, safet1y, and organoleptic properties together with enhance nutritional and functional properties due to transformation of substrates and formation of bioactive or bioavailable end-products. (Marco *et al*., 2017) [9]. LAB are generally auxotrophic for various water soluble vitamins even though some strains are able to produce water soluble vitamins like vitamin B_{12}. Some *Lactobacillus* strains have been exploited for the development of novel functional foods by producing/ releasing/ increasing some macronutrients and micronutrients (like vitamins) during fermentation (Bhushan *et al*., 2016) [1]. But, such novel strains need to be isolated from diverse ecological niches and characterized for their techno-functional attributes. There should be an appropriate screening strategy before exploiting them for the development of novel fermented functional foods (Capozzi *et al*., 2012) [3]. In a previous report from our lab, a novel genotypic strategy (based on *cbiK* gene) was employed for selection of *L. plantarum* strains with intracellular (Bhushan *et al*., 2016) [1] and *L. plantarum* and *L. rhamnosus* with extracellular B_{12} production potential (unpublished data). Hence, more screening studies are required to confirm vitamin B_{12} producing food-grade LAB.

From industrial point of view, it is essential to choose strains that perform well in fermentation and that oppose antagonistic conditions happening during the fermentation process. The LAB greatly differs in morphology, optimal growth and tolerance to temperature, salt and pH during fermentation process (George *et al*., 2018) [5]. Therefore, the strains should tolerate adverse conditions encountered during industrial processes, either during starter preservation and storage or during food processing in which abiotic stresses such as heat, cold, acidity, and high concentration of NaCl, sucrose or ethanol are common.

For environmental stress tolerance assay, the growth can be evaluated by the two most widely used methods i.e. the pour plate count method and Optical density (OD) measurements. Optical density (OD) estimations of microbial development are one of the most well-known procedures utilized in microbiology, to examine development under various dietary or stress situations. OD estimations are performed under the supposition that the OD value acquired is corresponding to the cell number i.e concentration of the sample. OD estimations have gotten synonymous with estimations of bacterial number (N) or concentration (C), as per the Beer-Lambert law. In any case, if cell size is relied upon to change fundamentally over the span of development of the microbial culture (for instance; development under different stress that instigate chains or clusters), OD estimations are never again reasonable and direct counting of N ought to be performed (Stevenson *et al*., 2016) [16]. Zapparoli, G. (2004) [19] showed that the variation of colony morphology and the different proteome expression of cells differentially aged were associated to stress resistance during starvation conditions. Biesta-Peters *et al*., 2010) [2] reported that OD measurements can be used to derive growth rates and lag times of bacterial cultures to investigate the effect of a large variety of pH values on growth parameters. However, plate counting will always remain a good method to locally investigate growth of cultures and to test the effect of specific growth conditions like modified atmospheres, which cannot be achieved in OD measurements, and will remain necessary for validation of new methods to establish parameters for growth.

Therefore, the purpose of this study was to confirm the presence of *CbiK* gene in reported extracellular vitamin B_{12} (unpublished data) with another primers which is assumed to be indicator of vitamin B_{12} production by LAB and measure the response of extracellular vitamin B_{12} producing strains to different environmental stresses (high temperature, cold temperature, ethanol and NaCl).

Materials and Methods

Materials

The research work was conducted in Techno Functional Starter Lab at ICAR-National Dairy Research Institute, Karnal, Haryana, India. Salt, sucrose used were from HIMEDIA, Mumbai, India, and Ethanol from Arihant Scientific, India. All PCR-related ingredients used were from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The *Lactobacillus* strains (*L. plantarum* NCDC958 and NCDC957, and *L. rhamnosus* NCDC953) 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. The cultures were maintained in glycerol (30%, v/v) at -20 °C.

Confirmation of reported B_{12} producing lactobacilli

The DNA was extracted (Pospeich and Newmann, 1995) [14] from the cultures isolated in our previous study and reported as extracellular vitamin B_{12} producing cultures (*L. plantarum* NCDC958 and NCDC957, and *L. rhamnosus* NCDC953) (unpublished data). All the DNA samples were confirmed through PCR amplification (Bio-Rad, Hercules, CA, USA) of *cbiK* gene (Bhushan *et al*., 2016) [1] using primers designed in this study shown in Table 1.

Table 1: Primers used for confirmation of cbiK gene

Sample source	Targeted gene/ region	Primer Sequences	Product size (bp)	Annealing Temperature (˚C)	Ref.
Genomic DNA	*cbiK*	F: GATGGGCGGCTATCCCAT TCTCCTT(25) R: CGGAACAGCGCATAGTG CTTTTACAACTTAT(31)	257	60	This study

Behavior of extracellular vitamin B_{12} producing cultures in environmental stress conditions

Understanding the stress response behavior of the culture is important to enhance its application in industrial fermentations.

Effect of temperature

The growth of three isolated *Lactobacillus* cultures was observed at different temperatures. A volume of 50µL saline washed cell suspension was inoculated into 5 mL of MRS broth and incubated at different temperatures (25 °C, 30 °C, 37 °C, 42 °C, and 50 °C). The growth was evaluated by measuring OD at 595nm and pour plating after 24 h of incubation.

Effect of NaCl

Three isolated *Lactobacillus* cultures were examined for various concentrations of NaCl. A volume of 50µL cell suspension washed with saline was inoculated into 5 mL of MRS broth containing different concentration of NaCl (0, 3%, 6%, 8%, and 10%). They were incubated at 37 °C for 24h. Their growth was measured by taking OD at 595 nm as well as by pour plating method.

Effect of sucrose

A volume of 50µL cell suspension of three isolated *Lactobacillus* cultures washed with saline was inoculated into 5 mL of MRS broth containing various concentrations of sucrose (0%, 10%, 20%, 30%, and 40% and incubated at 37 °C for 24h. Tolerance to different concentrations of sucrose was observed by measuring OD at 595 nm as well as by pour plating.

Effect of ethanol

The effect of ethanol on growth of three isolated *Lactobacillus* cultures was observed. 50µL saline washed cell suspensions was inoculated into 5 mL of MRS broth containing different concentration of ethanol (0, 2.5%, 5%, 10%, and 15%) and incubated at 37 °C. The growth was

evaluated by measuring OD595nm after 24 h of incubation as well as by pour plating.

Statistical analysis

The mean values and standard error generated from individual experiments were calculated and results were expressed in Mean ± SE. Besides use of Microsoft Office (2010) for tabulation and descriptive representation of data, GraphPad Prism (version 5) was used for Analysis of Variance (ANOVA) among values of statistical significance

Results and Discussion

The reliability of starter cultures in terms of functional properties (important for the development of functional foods), but also in terms of growth performance and robustness, has become essential for successful fermentations.

Confirmation of *cbiK* gene as screening strategy for vitamin B_{12} producing microorganism

All three previously reported extracellular vitamin B_{12} producing cultures (*L. plantarum* NCDC958 and NCDC957, and *L. rhamnosus* NCDC953) were found positive for the 257 bp *cbiK* gene from the primers designed in this study (Fig.1). The vitamin B_{12} production of the cultures is ascribed to the presence of whole B_{12} biosynthetic gene cluster, which encodes the enzymes required for the synthesis of vitamin B_{12} (Santos *et al*., 2008) [16], but for screening of vitamin B_{12} production ability has been linked with the specific detection of *cbiK* gene, which codes for important B_{12}-structural enzyme cobalt chelatase (Raux *et al*., 1997) [15]. In a previous study conducted by (Bhushan *et al*., 2016) [1] in our lab, a polyphasic sequential step was designed for screening of vitamin B_{12} producers i.e., to assess growth pattern of lactobacilli in B_{12}-free assay medium (VBAM) (first phase) and cobalt supplemented VBAM (second phase) and the presence of *cbiK* gene on their genomic DNA (third phase). The presence of *cbiK* gene on the genomic DNA was found to successfully selects B_{12}-producing strains contrary to first two phases (Bhushan *et al*., 2016) [1].

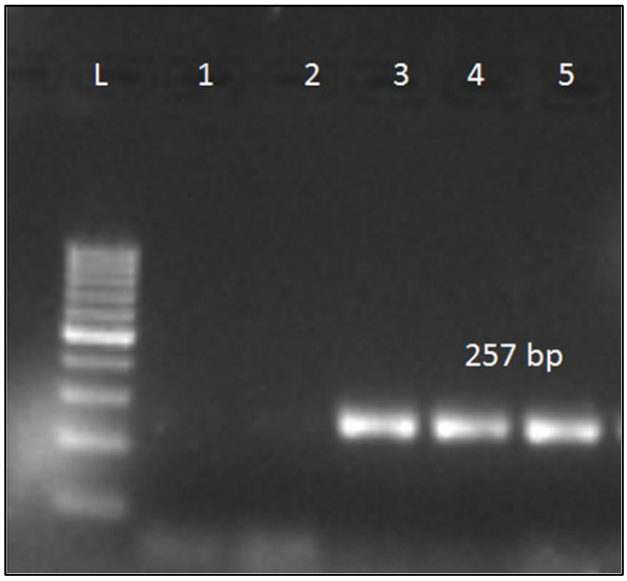

Fig 1: PCR based amplification of *cbiK* gene
L:Ladder; 1: negative; 2: Negative; 3: *L. plantarum* NCDC958, 4: *L. plantarum* NCDC957, 5: *L. rhamnosus* NCDC953

To be used as robust cultures, the cultures should have property to resist harsh conditions during the food fermentation process, such as extreme temperatures, ethanol and osmotic stresses to be encountered in food industries. The above mentioned three extracellular vitamin B_{12} producing cultures were examined for their tolerance to different incubation temperatures, various concentrations of NaCl, ethanol, and sucrose. The growth of each strain was observed after 24h of incubation by OD at 595nm (Masuda *et al*., 2012 [10]: Li *et al*., 2017) [8] as well as pour plating method.

Effect of temperature

Temperature is one of the major stresses that all microorganisms have to face. The exposure of culture to high temperature in industrial process might be deleterious to the cell. Hence, the culture should have potential to tolerate high temperature during fermentation process.

Table 2: Effect of temperature on growth of cultures (CFU/mL)

Cultures	Temperature (°C)				
	25	30	37	42	50
L. plantarum NCDC958	9.09±0.16	9.18±0.25	9.38±0.11	9.27±0.16	7.46±0.17
L. plantarum NCDC957	9.08 ±0.16	9.16±0.09	9.29±0.09	9.14±0.22	6.58±0.18
L. rhamnosus NCDC953	9.01±0.20	9.15±0.17	9.32±0.16	9.26±0.28	8.21±0.14

All three strains grew at higher (50 °C) temperatures, although not as good as that at 25 °C, 30 °C and 37 °C as absorbed by taking absorbance at 595nm **(**Fig.2). Similar results were obtained using pour plate count method (Table 2). In previous studies, Masuda *et al.* (2012) [10] reported that a few LAB strains grew at 45°C and Li *et al.* (2017) [8] reported three isolates showed growth at 45°C with an OD620 value of about 1.5. In this study all three isolates showed growth at 50 °C with no significant difference at $p<0.05$, compared by One-Way ANOVA. The capability of growth at 45 °C allows high temperature fermentation, which is very important for the brewing industry (O'Sullivan & Condon, 1997) [13].

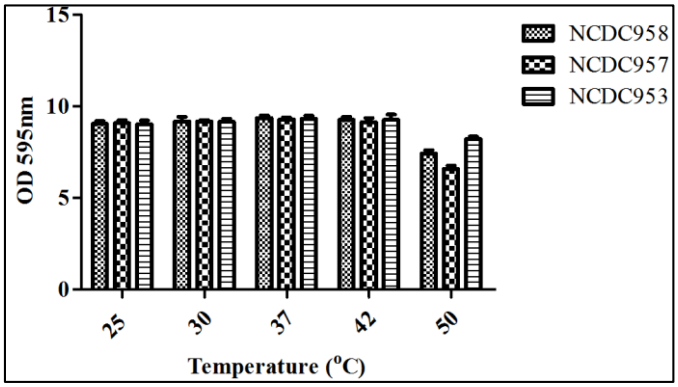

Fig 2: Effect of temperature

Effect of NaCl

Osmotic stress is often encountered by LAB in industrial process, such as cheese production and ripening, yoghurt preparation. Bacterial cell envelopes are permeable to water. In this way, an expansion in the osmolarity of the growth medium would bring about fast efflux of water from the cytoplasm. To hold water in the cell and, in this manner, to keep up turgor pressure, microscopic organisms have frameworks to aggregate explicit solutes that don't meddle with cell physiology. Such perfect solutes are either taken up

from the environment or newly incorporated in the cytoplasm; the uptake mechanism is found in most lactic acid bacteria (Csonka and Epstein, 1996) [4].

In this study, all three isolates (Fig.3), showed 100%, 80%, and 70% survival at 6%, 8%, and 10% NaCl concentration compared to 0% NaCl respectively at OD 595nm. Similar pattern was confirmed by plate count method (Table 3). In previous studies, (Masuda *et al*., 2012 [10]: Li *et al*., 2017) [8] reported that the most of LAB strains showed growth in osmotic concentration of NaCl, at 6%, but not at 8% or 10%. High osmotolerance would be a prerequisite of LAB strains for their commercial application. Because when lactic acid is produced by the strain during fermentation, alkali would subsequently be applied to prevent an excessive reduction in pH, which would result in the conversion of free acid into its salt form and thus increasing the osmotic pressure on the bacterial cells (Mohd Adnan & Tan, 2007) [11].

Table 3: Effect of NaCl on growth of cultures (CFU/mL)

Cultures	NaCl (%)				
	0	3	6	8	10
L. plantarum NCDC958	9.26±0.26	9.21±0.26	9.05±0.53	7.46±0.25	6.71±0.13
L. plantarum NCDC957	9.39±0.21	9.18±0.28	9.16±0.24	7.56±0.25	6.82±0.15
L. rhamnosus NCDC953	9.32±0.20	9.26±0.22	9.24±.064	7.62±0.22	6.93±0.11

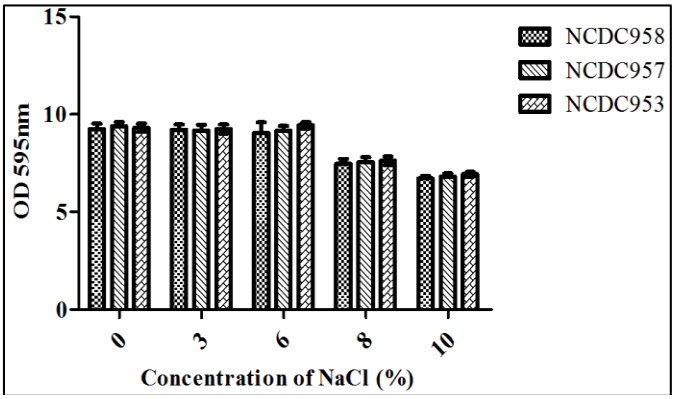

Fig 3: Effect of NaCl

Effect of sucrose

All the three isolates showed 100% survival up to 40% sucrose concentration by both OD at 595 nm (Fig.4.) and plate count method (Table 4).

Table 4: Effect of sucrose on growth of cultures (CFU/mL)

Cultures	Sucrose (%)				
	0	10	20	30	40
L. plantarum NCDC958	9.29±0.12	9.38±0.15	9.18±0.09	9.12±0.11	8.76±0.12
L. plantarum NCDC957	9.28 ±0.18	9.40±0.09	9.24±0.17	9.02±0.11	8.68±0.13
L. rhamnosus NCDC953	9.31±0.14	9.43±0.12	9.29±0.05	9.19±0.14	8.95±0.05

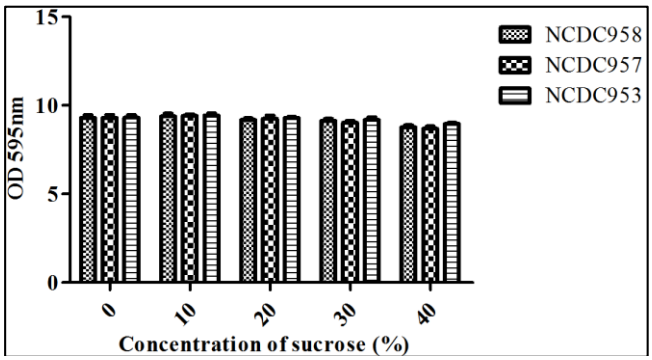

Fig 4: Effect of sucrose

Glaasker *et al*. (1998) [6] reported the effects on the growth of *L. plantarum* of raising high concentrations of KCl or NaCl and lactose or sucrose by nuclear magnetic resonance spectroscopy. There was more severe growth inhibition by salt stress than by equiosmolal concentrations of sugars reflects the inability of the cells to accumulate K^+ (or Na^+) to levels high enough to restore turgor as well as deleterious effects of the electrolytes intracellularly. This might be reason for the isolates used in this study to survive in high sucrose concentration but not in high NaCl concentration

Effect of ethanol

The capability of bacteria to grow at high concentrations of ethanol is necessary in the dairy as well as in wine industries. Masuda *et al*., (2012) [10] reported most of LAB strains showing tolerance to 5% of ethanol and only 3 of 74 cultures grew in broth with 15% of ethanol. In an another study (Li *et al*., 2017) [8] reported three *L. plantarum* isolates exhibited the ability to grow in 2.5%, 5%, 10% and 15% of ethanol were ~100%, 80%, 50% and 25% as that in 0% ethanol respectively. In our study, F2 and V7 showed 100%, 100%, 90% and 80% survival in 2.5%, 5%, 10% and 15% ethanol concentration as that in 0% ethanol respectively. Whereas F5 showed 100%, 100%, 100% and 90% survival in 2.5%, 5%, 10% and 15% ethanol concentration as that in 0% ethanol respectively (Table 5). This shows that these strains are good in tolerance to ethanol upto 15% concentration (Fig.5). Van *et al*. (2011) [18] described the molecular adaptation responses of *Lactobacillus plantarum* WCFS1 toward 8% ethanol exposure by using DNA microarrays. Ethanol exposure led to induced expression of genes involved in citrate metabolism and cell envelope architecture, as well as canonical stress response pathways controlled by the central stress regulators HrcA and CtsR.

Table 5: Effect of ethanol on growth of cultures (CFU/mL)

	Ethanol (%)				
Cultures	**0**	**2.5**	**5**	**10**	**15**
L. plantarum NCDC958	9.31±0.15	9.11±0.16	9.03±0.34	8.36±0.11	7.75±0.12
L. plantarum NCDC957	9.17±0.11	9.17±0.11	9.00±0.26	8.26±0.32	7.50±0.51
L. rhamnosus NCDC953	9.34±0.12	9.19±0.12	9.17±0.17	9.09±0.32	8.55±0.21

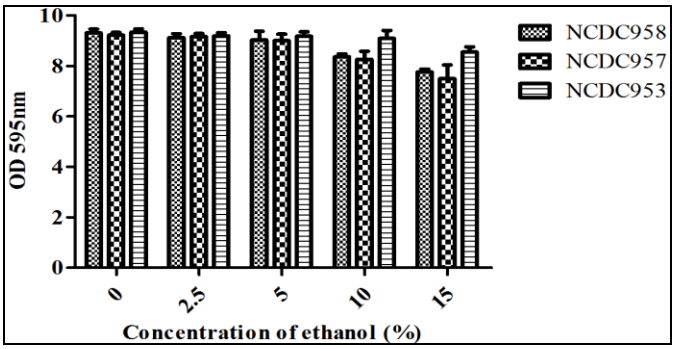

Fig 5: Effect of ethanol

Conclusion

The vitaminB_{12} producing potential is the strain specific attribute. So there should be appropriate screening methods for adding the functional properties of the culture in fermented food. The growth performance and robustness of the culture are key factors that determine the characteristics of the final products. We confirmed the genotypic-screening-protocol with designing new primer for the *cbiK* gene, a gene coding for enzyme cobalt chelatase among 30 gene clusters coding for vitamin B_{12} synthesis in our previously reported three extracellular vitamin B_{12} producing cultures (*L. plantarum* NCDC958, *L. rhamnosus* NCDC953, *L. plantarum* NCDC957). These cultures were found to be robust to tolerate the environmental stress (high temperature, ethanol, salt, and sucrose) along with having vitamin B_{12} producing properties. The robustness of vitamin B_{12} producing cultures i.e., to tolerate the environment stresses were studied for the first time with optical density (OD) measurements as well as plate count methods. Importantly, *L. rhamnosus* has first-time been reported for the environment stress tolerant extracellular vitamin B_{12}-producing culture. An appropriate screening of such techno-functional attributes and robustness of cultures are a few most important prerequisite steps for the manufacture of *in situ* vitamin B_{12} fortified food. The *in situ* fortification of vitamin B_{12} producing robust cultures in food would be better alternative to make vitamin B_{12} available to hosts. The further validation of the stress resistance mechanisms would endorse the use of culture for industrial fermented vitamin B_{12} fortified food on industrial scale.

Conflicts of interest

There is no conflict of interest declared.

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