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Enhanced vitamin B_{12} production using paneer whey as culture medium

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Abstract

Vitamin B_{12}, a water-soluble vitamin, plays a vital role in regulating metabolism in many organisms. However, its synthesis is restricted to limited bacteria and archaea. Therefore, industrial microbial fermentation is considered as a robust method to meet the demands of the vitamin B_{12}. *Lactobacillus reuteri* DSM 20016 is one of the vitamin B_{12} producers. In the present study, liquid paneer whey is used as the medium along with the supplements, glycerol, and cobalt chloride to enhance the vitamin B_{12} production. The effect of liquid paneer whey, yeast extract, glycerol, and cobalt chloride on the vitamin B_{12} production was further optimized using Box Behnken design. Under optimized conditions, the production of the vitamin B_{12} reached up to 213.449 $\mu g.L^{-1}$. The current study validated the feasibility of utilizing liquid paneer whey as a medium to produce high yield vitamin B_{12} by *Lactobacillus reuteri* DSM 20016.

Introduction

Vitamin B_{12}, a water soluble vitamin also known as cobalamin, is one of the largest complex vitamins widely used in medicine and also as nutrition supplements (Yu *et al.* 2015; Wang *et al.* 2020). The core of the vitamin B_{12} consists of a tetrapyrrolic corrin ring with a central cobalt atom, grouped with four nitrogen's, one nucleotide base group and one upper ligand. The recommended intake of the vitamin B_{12} for healthy adults is 2.4 µg/day. (Mohammed *et al.* 2014a). Vitamin B_{12} plays a significant role in the normal functioning of the nervous system, formation of blood, DNA synthesis and regulation, fatty acid synthesis and energy production. The deficiency of the vitamin B_{12} leads to complications such as pernicious anaemia, neurological dysfunctions such as inhibition of the physiological formation of the myelin sheath, altering correct nerve transmission (Mohammed *et al.* 2014b).

Plants, fungi, prokaryotes, and animals, including humans cannot synthesis vitamin B_{12} while synthesis is restricted to few classes of bacteria and archaea. Four main types of the vitamin B_{12} are cyanocobalamin (CN-Cbl), methylcobalamin (Me-Cbl), hydroxocobalamin (OH-Cbl), and deoxyadenosylcobalamine (Ado-Cbl). The cobalamins have different bio- and photo-chemistries. The predominant forms of vitamin B_{12} are Me-Cbl and Ado-Cbl and they are actively used as cofactors for enzymes methionine synthase and

methylmalonyl CoA mutase, while OH-Cbl and CN-Cbl are not used directly instead they are first converted into an active form of B_{12}. All forms of cobalamins are light sensitive, with Me-Cbl and Ado-Cbl identified as extremely labile, photodegrading into OH-Cbl in a matter of seconds after light exposure. All forms of cobalamin are converted to CN-Cbl to use for pharmacological and commercial purposes, because it is the most stable form of the vitamin B_{12} in the presence of light (Heal *et al.* 2014; Mohammed *et al.* 2014b).

When the consumption of animal foods is very low or absent and scarce presence in plant foods, an adherent importance to the molecule is created, making it essential, either through supplements or fortified foods. This deficiency is common among vegetarians. Hence, the production of the vitamin B_{12} has received a great attention due to increasing global requirements. Production of cobalamin relies solely on microbes since chemical methods remain economically not feasible due to the technical complexity of the synthetic process. The natural process of the vitamin B_{12} synthesis consists of approximately 30 enzymes mediated steps proceeding either through aerobically evident in *Pseudomonas denitrificans*, or anaerobically as witnessed in *Bacillus megaterium*, *Propionibacterium shermanii*, *Salmonella typhimurium*, and *Lactobacillus reuteri* (Mohammed *et al.* 2014a).

Lactobacillus reuteri is a Gram-positive, hetero-fermentative lactic acid bacterium, prevalent throughout the gastrointestinal tract of humans and other animals (Santos *et al.* 2011; Walter *et al.* 2011). *Lactobacillus reuteri* is known to has probiotic properties (Taranto *et al.* 2003) and a functionally active B_{12} biosynthetic gene cluster (Santos *et al.* 2009). The bacteria were also able to secrete reuterin, a broad-spectrum antimicrobial agent produced during anaerobic sugar-glycerol co-fermentation (Talarico *et al.* 1988; Talarico and Dobrogosz 1989).

Whey, a by-product of the dairy industry is rich in lactose, proteins, and minerals. Lactose and protein content vary from 39 to 45 $g.L^{-1}$ and 9 – 14 $g.L^{-1}$ respectively. About 0.7 % (w/v) of the total solid content of whey consists of whey proteins. The notable whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins (IgG) (Kumar *et al.* 2012; Priyanka and Rastogi 2018; Kaur *et al.* 2020). The global production of whey is around 160 million tons per year and out of the total production of whey, only 70 % is used in the production of various products, while 30 % is disposed of in rivers and seas. Regardless of all, whey is one of the major pollutants in the food processing industry. A million tons of whey are disposed into rivers, lakes, other water bodies which possess serious threat to the ecosystem due to its high biological and biochemical oxygen demand. In India, the production of whey is scattered, unorganized and the production levels vary from small (20 – 50 L/day) to large scale (50,000 – 1,000,000 L/day) hence, may not be processed at one location (Kakan *et al.* 2018). Hence, it is crucial to devise strategies for proper utilization of whey.

Many optimization studies have been carried out using experimental design techniques such as Plackett-Burman Design, Box–Behnken Design and Central Composite Design have been widely used in the literature (Şensoy *et al.* 2020). Box-Behnken Design, a response surface methodology statistical design was widely used to explore the correlation between process variables and the responses using minimal experiments (Jeganathan *et al.* 2014). The current study aimed to investigate the practicability to use paneer whey as a growth medium for production of the vitamin B_{12} and to optimize the media components to achieve the maximum vitamin B_{12} production using Box-Behnken design.

Experimental

Materials

Vitamin B_{12} (Cyanocobalamin, 99.9 % pure), HPLC water, and methanol was purchased from Sigma Aldrich Chemicals Private Limited (Bangalore, India). All other chemicals and reagents used were of analytical grade.

Whey procurement and characterization

Liquid unprocessed paneer whey (LPW) was obtained from a cottage dairy industry in Chennai,

Tamilnadu. LPW was analysed for pH and water activity. The protein and ash content were determined according to Indian Standard method IS 4684-1975 (1976). Fat content was estimated by the AOAC method 922.06. The carbon and nitrogen content were determined using the CHNS analyzer at CSIR-CLRI, Chennai.

Strain and culture conditions

The bacterial strain used in the study was *L. reuteri* DSM 20016, acquired from Metabolic Engineering laboratory, Anna University, Chennai. To obtain a working culture, from the frozen stock, 100 µL of the thawed culture was transferred to 10 mL of the MRS broth (de Man *et al*. 1960) and incubated for 24 h at 37 °C. Daily transfer to fresh medium was performed to maintain the cell viability prior to use. Cells were cultivated in non-stirred batch cultures under anaerobic conditions (95 % N_2 and 5 % CO_2) unless stated otherwise.

Preparation of growth medium

Production of vitamin B_{12} by *L. reuteri* DSM 20016 was done in LPW medium consisting of 20 g LPW, 5 g yeast extract, 1 g polysorbate 80,2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5). The medium composition was adapted from the MRS medium in which LPW was replaced as a carbon source instead of dextrose. Glycerol and cobalt chloride were used as supplements to enhance the vitamin B_{12} production. The composition of the media was modified according to the experiments.

Optimization of vitamin B_{12} production

The growth kinetics of *L. reuteri* DSM 20016 was monitored in MRS and LPW medium at 37 °C. The preliminary screening assessed the effect of varying individual components of LPW - yeast extract (2.5 – 15 $g.L^{-1}$), polysorbate 80 (1 – 6 $g.L^{-1}$), LPW (20 – 120 $g.L^{-1}$). The effect of varying levels enhancers glycerol (1 – 8 $g.L^{-1}$) and cobalt chloride (1 – 8 $g.L^{-1}$) were studied individually on the production of vitamin B_{12} was also evaluated. The other components were maintained at a fixed level. The starter culture of OD_{600} = 0.961 (corresponds to CFU =1.14 × 10^4 $CFU.mL^{-1}$) was used as inoculum. All experiments were conducted in duplicates and average values were reported. The SPSS 11.5 software package was used for statistical evaluation of data. The difference between the runs was evaluated using Duncan's test at 5 % significance level.

Response surface methodology

As the subsequent step, the response surface methodology (RSM) was conducted by applying Box–Behnken design to study the optimal medium formulation and the interactions between the factors for the vitamin B_{12} production. Box-Behnken designs a three-level second-order design developed to fit the second-order response surface model (Vellaisamy Singaram *et al.* 2021). The factors of Box Behnken design along with the levels are as follows – LPW (20 – 80 $g.L^{-1}$), yeast extract (2.5 – 5 $g.L^{-1}$), glycerol (1 – 3 $g.L^{-1}$) and cobalt chloride (1 – 3 $g.L^{-1}$). The model was developed using a Design Expert (Stat-Ease) software version 11.1. The design matrix with actual values and experimental results are shown in Table 2. The experimental results were fitted with a second order polynomial function (Eq. 1):

$$Y = \beta_O + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \cdot \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + e_{ij} \quad (1)$$

where Y is the predicted response, k is the number of factors, x_i and x_j are the coded variables; β_0 is the offset term; β_i, β_j, and β_{ij} are the first-order, quadratic, and interaction effects, respectively; i and j are the index numbers for factor; and e_{ij} is the residual error.

The fit quality of the model was evaluated by R^2 and analysis of variance (ANOVA). The lack of fit was used for controlling the statistics adequacy and efficiency of the model. Statistical testing of the model was done by Fisher's statistical test (Kumar *et al.* 2012).

Extraction and analysis of vitamin B_{12}

The vitamin B_{12} (Cyanocobalamin) was quantified using High performance liquid chromatography. Hydroxocobalamin is a natural form of cobalamin, formed in the bacterial cell. Since it is highly unstable, it is converted to cyanocobalamin using KCN (Hajfarajollah *et al.* 2015). For high-performance liquid chromatography (HPLC) analysis, the cell culture broth (40 mL) was centrifuged (9,000 × g for 10 min at 4 °C) to harvest the cells. The supernatant was discarded, and the pellet was washed with 10 mL 0.2 M potassium phosphate buffer (pH 5.5), centrifuged (10,000 × g for 15 min) and re-suspended in 1 mL of 0.2 M potassium phosphate buffer (pH 5.5) containing 0.1 % potassium cyanide. In order to lyse the cells for release of the vitamin B12, the re-suspended cells were vortexed and autoclaved (121 °C, 15 min). Finally, the samples were vortexed again and centrifuged (10,000 × g, 15 min). The supernatant was filtered (0.22µm syringe filter) and analysed with HPLC (Hugenschmidt *et al.* 2010).
A Shimadzu chromatography system with C18 column (Shodex C18-4E, I.D. × length, mm - 4.6 × 250) and UV-Vis detector was used for the analysis of Vitamin B_{12}. The UV detection wavelength, flow rate of a mobile phase, oven temperature, and injection volume were set to 361 nm, 1 $mL.min^{-1}$, 30 °C, and 20 µL, respectively. The mobile phase was a mixture of HPLC water: Methanol (50 : 50). An external standard (Cyanocobalamin) was used for calibration. Each sample was injected twice, and the mean values were reported.

Results and Discussion

Characterization of liquid paneer whey

The compositional characteristics of LPW are shown in Table 1. The proximate composition of LPW was 0.44g/100g protein, 4.91 % lactose, 0.16g/100g ash. The LPW has 97.63 % moisture content, and it is devoid of fat. The carbon and nitrogen contents of LPW were determined as 52.941 % and 1.187 %, respectively. The water activity value suggests LPW as a suitable medium for bacterial growth. Similar observations on the composition of paneer whey have been reported in the literature (Ranvir and Adil 2018; Singh *et al.* 2019).

Table 1. Compositional analysis of LPW.

S. No.	Parameters	Value
1	Moisture [%]	97.63
2	Ash [g/100g]	0.16
3	Protein [g/100g]	0.44
4	Fat [g/100g]	0.001
5	Lactose [%]	4.91
6	Water activity [a_w]	0.98
7	pH	5.73
8	Density [$g.L^{-1}$]	1.0178

Growth analysis

The growth curve of *L. reuteri* DSM 20016 strain in MRS and LPW medium was studied (Fig. 1 and Fig. 2).

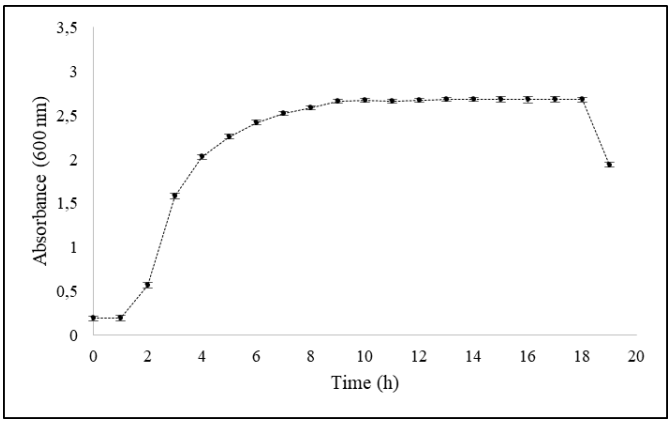

Fig. 1. Growth curve of *L. reuteri* DSM 20016 in MRS medium (Experiments were conducted in triplicates and the standard deviation was denoted as error bars).

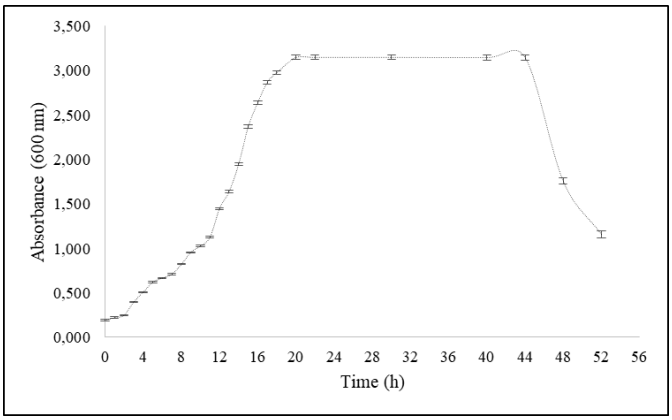

Fig. 2. Growth curve of *L. reuteri* DSM 20016 in LPW medium (Experiments were conducted in triplicates and the standard deviation was denoted as error bars).

The exponential phase in the MRS medium reached a peak at 8 h and the stationary phase existed till 18 h. The carbon source in the MRS medium is glucose, which is easily metabolized by *Lactobacillus sp*. After 18 h the amount of nutrients will be depleted, resulting in cell death. LPW was reported to be an excellent liquid carrier on the basis of bacterial shelf-life and presence of lactose and proteins which can serve as carbon and nitrogen sources for microorganisms (Tan and Li 2018; Prakash and Arora 2020). In the LPW media the exponential phase reached peak at 18 h and the stationary phase extended till 44 h. The delayed cell growth in the LPW media might be due to the complex nature of lactose since bacterial β-galactosidase activity is necessary to metabolize lactose. *L. reuteri* possesses low proteolytic activity and therefore supplementation with extra nitrogen sources might improve growth (Jantzen *et al.* 2013). The number of cells obtained in MRS and LPW medium at 18 h is 1.46×10^7 CFU.mL^{-1} and 2.41×10^9 CFU.mL^{-1} respectively.
Since the vitamin B_{12} is a primary metabolite, the sampling time point in the experiment is chosen as 18 h.

Effect of media components on vitamin B_{12} production

The effect of media components – yeast extract, polysorbate 80, and LPW on the vitamin B_{12} production was studied and the results are presented as follows.

Yeast extract

Yeast extract is commonly used for the growth of lactic acid bacteria due to their composition in amino acids and vitamins, which is necessary for the cell growth. Yeast extract is the most common nitrogen source employed in the published studies concerning *L. reuteri* (Couvreur *et al.* 2017; Ichinose *et al.* 2020; Nguyen *et al.* 2021). In the present study, the effect of varying the amount of yeast extract (2.5 g – 15 g) on the vitamin B_{12} production was analysed. The other components of LPW media are as follows – 20 g LPW, 1 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5). As shown in Fig. 3, the increase in amount of yeast extract from 2.5 g.L^{-1} – 5 g.L^{-1} significantly improved ($P < 0.05$) the vitamin B_{12} production from 25.578 ± 0.282 µg.L^{-1} to 32.728 ± 0.215 µg.L^{-1}. Further increase in the amount of yeast extract reduced the vitamin B_{12} production. The lowest vitamin B_{12} (14.861 ± 0.037 µg.L^{-1}) was obtained at 15 g.L^{-1} of yeast extract. The negative effect of yeast extract might be due to toxicity of yeast extract at higher concentration which in turn decreases the cell concentration. Similar results on the negative effect of yeast extract on the growth of *Lactobacillus* have been reported in the literature (Ghaly *et al.* 2003; Miloud *et al.* 2017). The optimal concentration of yeast extract for the production of the vitamin B_{12} was found to be 5 g.L^{-1}.

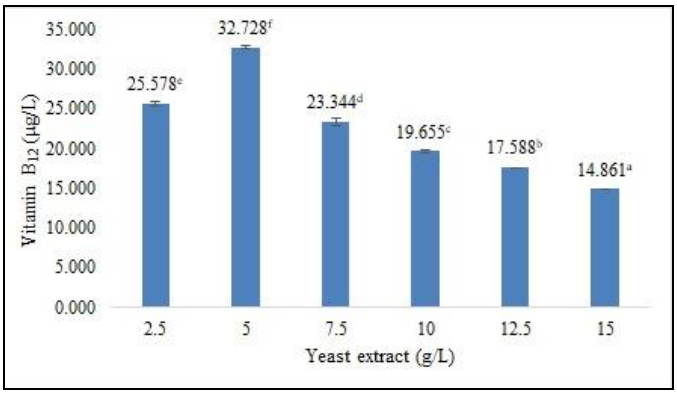

Fig. 3. Effect of varying amount of yeast extract on vitamin B_{12} production. Values with different superscripts are significantly different ($P < 0.05$).

Polysorbate 80

To further investigate the influence of polysorbate 80 on the vitamin B_{12} production, polysorbate 80 concentrations from 1 to 6 g.L^{-1} were used, and other media components are as follows – 20 g LPW, 5 g yeast extract, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5). Polysorbate 80, a surfactant, is a crucial growth factor in culture media for *Lactobacilli* as it brings unsaturated fatty acids that allow reducing intracellular energy consumption (Nguyen *et al.* 2021). As shown in Fig. 4, highest and lowest vitamin B_{12} production was obtained at 3 g.L^{-1} (35.796 ± 0.012 µg.L^{-1}) and 6 g.L^{-1} (30.846 ± 0.058

µg.L^{-1}). Higher concentrations of polysorbate 80 decreased the vitamin B_{12} production because polysorbate 80 became toxic at higher concentration due to the destruction of the cell membrane structure and/or loss of the cell membrane function caused by the solubility of lipid bilayer by the surfactant (Qi *et al.* 2009). Hence, the optimal concentration of polysorbate 80 to produce the vitamin B_{12} was found to be 3 g.L^{-1}.

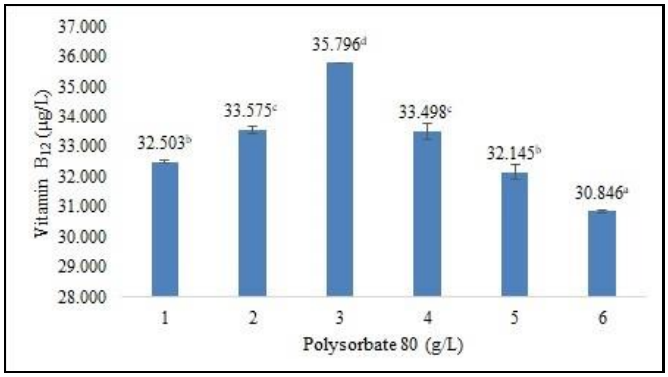

Fig. 4. Effect of varying amount of polysorbate 80 on vitamin B_{12} production. Values with different superscripts are significantly different ($P < 0.05$).

Paneer whey

For analysis of the effect of LPW on the vitamin B_{12} production, the yeast extract and polysorbate 80 levels were set at 5 g.L^{-1} and 3 g.L^{-1}. The level of LPW was varied between 20 to 120 g.L^{-1} and other components were fixed as follows – LPW medium consisting of, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5). As shown in Fig. 5, the vitamin B_{12} production was significantly increased with the increase of LPW percentage from 20 to 80 g.L^{-1}. The highest vitamin B_{12} was achieved at cultures with 80 g.L^{-1} LPW. It has been reported that LPW is rich in various components, such as carbohydrate, vitamins and organic acids (Patowary *et al.* 2016). Due to the complex nutritional requirements of the *Lactobacillus*, their survival is favoured in the presence of increased carbohydrate, protein, and vitamin sources (Gomes *et al.* 2015). When further increasing the LPW amount into 100 g.L^{-1} or more, it drastically decreased the production of the vitamin B_{12}. Hence, the highest vitamin B_{12} produced was 65.179 ± 0.093 µg L^{-1} and the optimal concentration of LPW was 80 g.L^{-1}. After determining the optimal concentration of yeast extract, polysorbate 80 and LPW, the effect of glycerol and cobalt chloride on the vitamin B_{12} production was analysed.

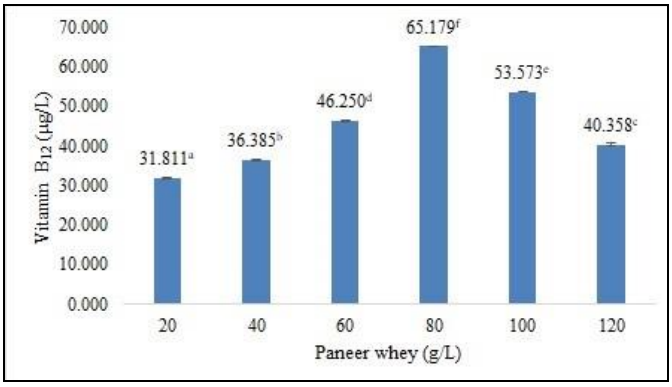

Fig. 5. Effect of varying amount of LPW on vitamin B_{12} production. Values with different superscripts are significantly different ($P < 0.05$).

Effect of glycerol on vitamin B_{12} production

The effect of glycerol was analysed in a medium containing 80 g LPW, 5 g yeast extract, 3 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5) with varying amounts of glycerol (1 g.L^{-1} to 8 g.L^{-1}). *L. reuteri* was not capable of growing in glycerol as a sole carbon and energy source, even in the presence of complex ingredients of MRS broth (Gopal Ramakrishnan *et al.* 2015). During glycerol fermentation of *L. reuteri*, glycerol serves only as an external hydrogen acceptor (Diraviam Sriramulu *et al.* 2008). Glycerol plays an important role in the growth of cells and acts as an inducer for vitamin B_{12} production. The genes responsible for the synthesis of cobalamin are cobT and cbiA. The CbiA catalyzed amidations to a side chain of cobyrinic acid in the biosynthesis of cobalamin (vitamin B_{12}) from uroporphyrinogen III. The lower ligand of cobalamin is covalently linked to a phosphoribosyl moiety through an alpha-glycosidic bond formed by the CobT enzyme. However, CobT was reported that it is responsible for the production of analogues. The expression of cbiA and cobT was reported to raise significantly with an increase of glycerol supplementation (Crofts *et al.*

2014; Gu *et al.* 2015). In the present study, the highest and lowest vitamin B_{12} production was obtained at 3 $g.L^{-1}$ (95.704 ± 0.022 $\mu g.L^{-1}$) and 8 $g.L^{-1}$ (29.640 ± 0.001 $\mu g.L^{-1}$) (Fig. 6). The higher concentrations of glycerol had a negative effect on vitamin B_{12} production. It was reported that the vitamin B_{12} production was enhanced with the supplementation of glycerol but high concentrations of glycerol supplementations inhibited the growth of cells which might be due to the activity of glycerol dehydratase inhibited by a quorum sensing of reuterin (Bauer *et al.* 2010; Gu *et al.* 2015). Therefore, the optimal concentration of glycerol to produce the vitamin B_{12} was found to be 3 $g.L^{-1}$.

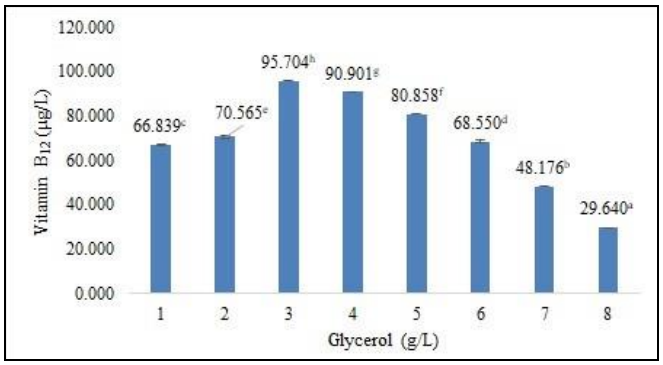

Fig. 6. Effect of varying amount of glycerol on vitamin B_{12} production. Values with different superscripts are significantly different ($P < 0.05$).

Effect of cobalt chloride on vitamin B_{12} production

Lastly, the effect of cobalt chloride was analysed in medium containing 80 g LPW, 5 g yeast extract, 3 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulphate, 0.05 g manganese sulphate, and 2 g dipotassium hydrogen phosphate in 1 L of distilled water (pH 6.5) with varying concentrations of cobalt chloride from 1 $g.L^{-1}$ to 8 $g.L^{-1}$. Glycerol was not included in the media to study the individual effect of cobalt chloride on the vitamin B_{12} production. Cobalt chloride is a part of the metabolic pathway of the vitamin B_{12} production. The availability of cobalt chloride ions in the culture medium is essential to the vitamin B_{12} biosynthesis because it is a central component of the corrinoid ring (Burgess *et al.* 2009). The amount of $CoCl_2.6H_2O$ is significant in affecting the final concentration of the vitamin B_{12} (Hajfarajollah *et al.* 2015). As shown in Fig. 7, maximum vitamin B_{12} production of 147.633 ± 0.367 $\mu g.L^{-1}$ was achieved at the concentration 3 $g.L^{-1}$. Similarly, results of the increasing concentrations of cobalt chloride in enhancing the vitamin B_{12} production in *Bacillus megaterium* and *Propionibacterium freudenreichii subsp. shermanii* ATCC 13673 has been reported in literature (Mohammed *et al.* 2014a; de Assis *et al.* 2020). However, further increase in the concentration to 4 $g.L^{-1}$ or more depleted the vitamin B_{12} production.

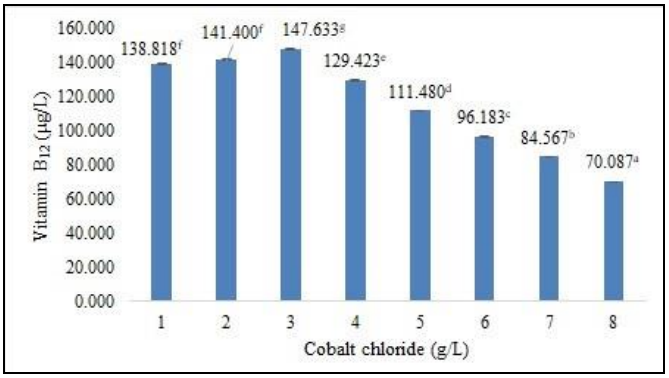

Fig. 7. Effect of varying amount of cobalt chloride on vitamin B_{12} production. Values with different superscripts are significantly different ($P < 0.05$).

Response surface methodology

The components in LPW medium, sodium acetate, K_2HPO_4, ammonium citrate, $MgSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ were considered not to have a significant effect on the biomass production (Griet *et al.* 2018) and based on the single variable experiments, polysorbate 80 doesn't induce a significant increase in the vitamin B_{12} production. Hence, in the present study, the effect of four factors – paneer whey (A), yeast extract (B), glycerol (C) and cobalt chloride (D) on the vitamin B_{12} production were optimized using Box Behnken design. The design matrix along with the experimental values is in Table 2. The production of the vitamin B_{12} varied from 54.863 $\mu g.L^{-1}$ to 213.449 $\mu g.L^{-1}$ and the maximum production was achieved at the following conditions – Paneer whey (80 $g.L^{-1}$), yeast extract (5 $g.L^{-1}$), glycerol (2 $g.L^{-1}$) and cobalt chloride (2 $g.L^{-1}$). A modified quadratic model was developed to model the experimental data.

Table 2. Box Behnken design matrix with actual values and experimental results.

Std	Run	LPW (A)	Yeast extract (B) [g.L^{-1}]	Glycerol (C) [g.L^{-1}]	Cobalt chloride (D) [g.L^{-1}]	Vitamin B_{12} [µg.L^{-1}]
1	2	20	2.5	2	2	54.8634
3	20	20	5	2	2	80.2672
9	17	20	3.75	2	1	72.616
11	25	20	3.75	2	3	81.4221
17	16	20	3.75	1	2	76.8487
19	13	20	3.75	3	2	80.4883
5	28	50	3.75	1	1	108.055
6	7	50	3.75	3	1	121.639
7	24	50	3.75	1	3	124.885
8	18	50	3.75	3	3	129.369
13	26	50	2.5	1	2	85.7037
14	14	50	5	1	2	134.839
15	15	50	2.5	3	2	93.8982
16	21	50	5	3	2	141.774
21	27	50	2.5	2	1	83.4567
22	10	50	5	2	1	131.367
23	23	50	2.5	2	3	91.8839
24	29	50	5	2	3	143.159
25	22	50	3.75	2	2	109.055
26	12	50	3.75	2	2	109.609
27	4	50	3.75	2	2	110.545
28	1	50	3.75	2	2	110.748
29	3	50	3.75	2	2	110.532
30	30	50	3.75	2	2	110.58
2	19	80	2.5	2	2	138.321
4	9	80	5	2	2	213.449
10	6	80	3.75	2	1	175.385
12	8	80	3.75	2	3	189.326
18	5	80	3.75	1	2	176.45
20	11	80	3.75	3	2	190.726

The analysis of variance (ANOVA) results for the model are presented in Table 3.

Table 3. ANOVA for reduced quadratic model.

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	44259.68	12	3688.31	3300.21	< 0.0001	Significant
A-LPW	33830.15	1	33830.15	30270.42	< 0.0001	
B-Yeast extract	7337.31	1	7337.31	6565.25	< 0.0001	
C-Glycerol	217.71	1	217.71	194.80	< 0.0001	
D-Cobalt chloride	379.98	1	379.98	340.00	< 0.0001	
AB	618.12	1	618.12	553.08	< 0.0001	
AC	28.28	1	28.28	25.31	0.0001	
AD	6.59	1	6.59	5.90	0.0265	
CD	20.70	1	20.70	18.52	0.0005	
A^2	1443.02	1	1443.02	1291.18	< 0.0001	
B^2	47.83	1	47.83	42.80	< 0.0001	
C^2	273.95	1	273.95	245.13	< 0.0001	
D^2	158.43	1	158.43	141.76	< 0.0001	
Residual	19.00	17	1.12			
Lack of Fit	16.67	12	1.39	2.98	0.1183	Not significant
Pure Error	2.33	5	0.4663			
Cor Total	44278.68	29				

The model was found to be significant ($P < 0.05$) and the statistical significance of the model was analysed using the Fisher test. In the current study, the significant model terms are found to be A, B, C, D, AB, AC, AD, CD A^2, B^2, C^2 and D^2. The Lack of Fit F-value of 2.98 implies the lack of fit is not significant.The high value of the coefficient of determination (0.9996) indicates the model is significant. In addition to this, the predicted R^2 (0.9983) and adjusted R^2 (0.9993) values must be less than 20 % which further suggests the adequacy of the model.

The coded equation for the modified quadratic model is given as follows Eq. 2:

$$\text{Vitamin } B_{12} \ (\mu g.L^{-1}) = +110.18 + 53.10\ A + 24.73\ B + 4.26\ C + 5.63\ D + 12.43\ AB + 2.66\ AC + 1.28\ AD - 2.27\ CD + 14.51\ A^2 - 2.64\ B^2 + 6.32\ C^2 + 4.81\ D^2 \quad (2)$$

The influence of the process variables on the vitamin B_{12} production was studied with the help of 3D surface plots (Fig. 8). The vitamin B_{12} production was increased with respect to increasing LPW and yeast extract concentrations. The interactive effect between LPW and yeast extract was positive. The interactive effect of glycerol and cobalt chloride on the vitamin B_{12} production was negative. The negative effect might be due to the toxic nature of the compounds.

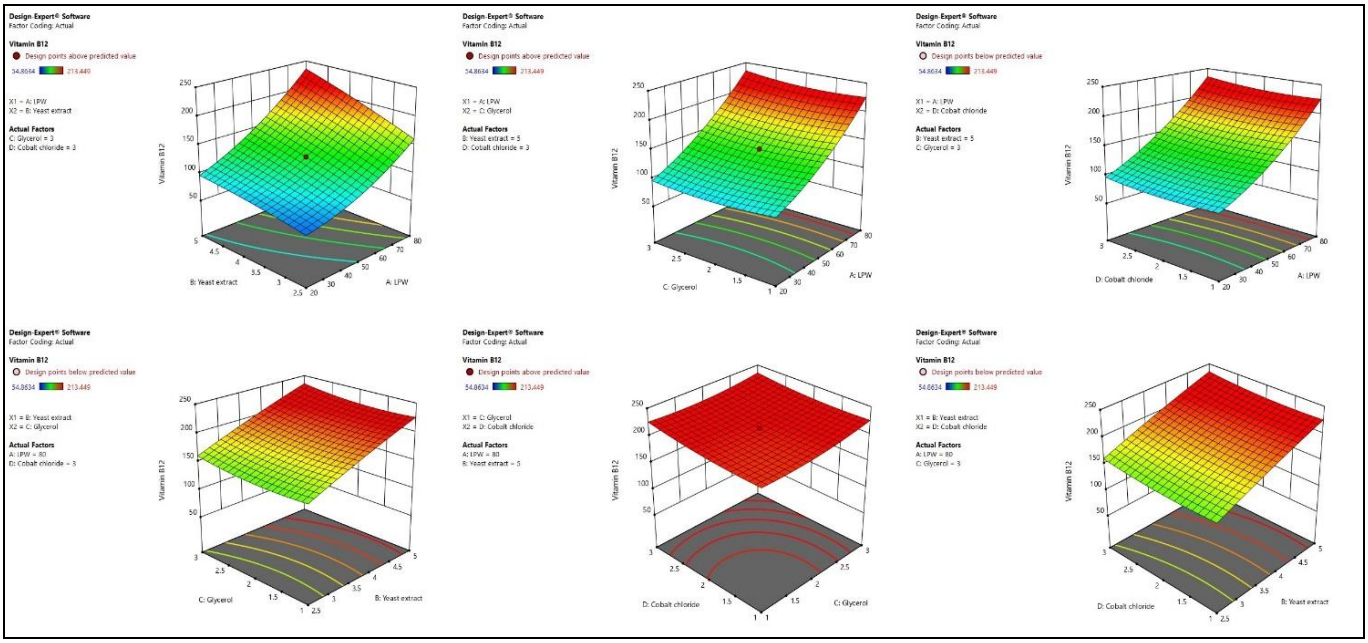

Fig. 8. 3D Surface plots showing the influence of process variables on the vitamin B12 production.

Conclusion

From the current study, it could be concluded that paneer whey is an excellent substrate for the vitamin B_{12} production. The concentrations of paneer whey, yeast extract, glycerol and cobalt chloride has most distinct effects on the biosynthesis of the vitamin B_{12} by *L. reuteri* DSM 20016. Response surface methodology was used to optimize the process variables and the optimal condition for the vitamin B_{12} production was found to be Paneer whey (80 $g.L^{-1}$), yeast extract (5 $g.L^{-1}$), glycerol (2 $g.L^{-1}$) and cobalt chloride (2 $g.L^{-1}$). The maximum of the vitamin B_{12} production obtained was 213.449 $\mu g.L^{-1}$. The current study proposes valuable insights on cost effective carbon source to produce the vitamin B_{12}.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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