International Journal of Recent Advances in Multidisciplinary Topics
Volume 2, Issue 11, November 2021
https://www.ijramt.com | ISSN (Online): 2582-7839

Determination of Substrate Dependent Enhancement of Vitamin B12 Synthesis during Mixed Culture Fermentation of Soybean, Bengal Gram and Rice with Microbial Species

Swasti Srivastava*
Department of Biosciences and Biotechnology, Chattrapati Shahu Ji Maharaj University, Kanpur, India

***Abstract*: Since vitamin B12 is an exception to other vitamins being present only in animal foods, hence, it is an elusive nutrient for strict vegetarians. The aim of present study was enhancement of vitamin B12 synthesis by fermenting combination of substrates in presence of different microbial inoculates. Rhizopus was used as main fermenting microbe alone or in combination with either Pseudomonas or Lactobacillus or both. Vitamin B2, B6, B12 content was quantified indirectly by using second order derivative spectrophotometry at λmax=267nm for B2, λmax=309nm for B6, λmax=361nm for B12, combined with statistical analysis based on regression. The studies concluded that soyabean+ Bengal gram substrate combination and the mixed culture of Rhizopus+Pseudomonas+Lactobacillus was most suitable for B complex vitamin production by fermentation. The study is first of its kind where different substrate combinations have been used vis-a-vis different microbial mixed cultures for increased synthesis of elusive vitamin B12.**

***Keywords*: B12, Derivative Spectrophotometry, Regression Analysis**

1. Introduction

The vitamins of B-complex are required by humans for energy metabolism, neurotransmitter synthesis, as coenzyme for enzymes of metabolic pathways and affect growth, mental activity, visual and psychomotor functions [1], [2]. B2 and B6 are the psychomotor vitamins responsible for proper neural activity. The deficiency of these vitamins results in impaired cognitive function, cardiovascular diseases, increased risk of cancer and many more similar complications [2], [3]. B12 acts as a cofactor for metabolic enzymes in the cytoplasm as well as mitochondria and affects key metabolic functions of other enzymes [4], [5]. It is required for synthesis of myelin, phospholipids, proteins and neurotransmitters [6]. Thus, vitamin B12 is physiologically indispensable and its deficiency may impair these functions. Vitamin B12 like other B complex vitamins is water soluble and needs to be replenished in body by dietary supplements at a recommended concentration of 2.4μg/day for adults [7]. In the absence of any supplementary diet, the deficiency of these may lead to angular cheilitis, Dermatologic and neurologic disorders and various other biological malfunctions [8], [9]. The compromised physiological absorptive processes and lack of adequate diet supplementation are assumed to be main cause of B12 deficiency [10], [11], [12]. Due to rise of animal ethics concern, a trend reversal to vegetarianism may also contribute to B12 deficiency [13].

The biosynthesis of Vitamin B12 is an oxygen labile process consisting of about 30 enzymatic reactions and intermediates which makes prokaryotes most likely candidates for B12 synthesis as against eukaryotes [14], [15]. Thus, B12 supplementation in vegans has been a challenge for researchers. Most of the vitamin B complex vitamins can be supplemented in diet from dairy, vegetable, fruit and other animal food products except B12 which is solely present in animal food sources [16], [17]. Most of the vegan food sources are fortified with B12 to accomplish the recommended dietary intake. Thus, B12 is being industrially produced either by chemical synthesis or microbial fermentation by Propionibacterium and Pseudomonas species [18], [19]. However, due to number of steps required for chemical synthesis, fermentation methods are preferred [20]. According to some studies of the food ingredients, fermentation naturally leads to changes in nutrient contents like vitamins, essential amino acids proteins and antinutrients with improvement in food texture, flavour and aroma depending on substrates and fermenting microbes [21].

In recent years many fermentable substrates have been used for enhancement of synthesis of vitamin B12 but use of substrate combinations is still not found [22], [23], [24]. Most studies for enhancement of B-complex vitamins either use single substrates with mixed cultures or multiple substrates with pure culture. The aim of the study was to evaluate effect of combinations of substrates and fermenting microbes on vitamin B12 content. Thus the study is an exception as different substrate combinations have been used with different combination of microbial cultures.

*Corresponding author: srivastava25s@gmail.com

2. Material and Methods

1) Isolation and Characterization of Pure Culture of Rhizopus species

Pure cultures of Rhizopus spores were isolated from soybean seeds, soaked and incubated between Hibiscus leaves at 36-42ºC[25]. The fungal mycelium formed within 3 days followed by sporulation at seven day interval. The species colony was selected on the basis of morphological characteristics like color (white) and mycelium structure (wooly). The identity was further ascertained by cotton-blue lacto-phenol blue staining of the mycelium. The selected colonies were cultured on Potato Dextrose Agar (50 ml potato extract, 5g Dextrose, 5g Agar and double distilled water (DDW) to make up final volume 250 ml) and transferred to the same media again and again for production of fungal biomass. The pure cultures were regularly maintained on Potato Dextrose Agar at 4ºC and sub-cultured every month. The spores of the Rhizopus were used as inoculums for substrate fermentation.

2) Isolation and Characterization of Pure Culture of Pseudomonas species

Pseudomonas species were isolated from nodulated plant roots. The samples from root nodule were suspended in DDW and serially diluted from 10-1 to 10-10 dilutions. The dilutions were poured on Pseudomonas Fluorescent Agar (3.8g/100 ml Pseudomonas Fluorescent Agar, 1ml glycerol and DDW to make up final volume to 250 ml) and incubated in a B.O.D. incubator at 32ºC for 3-4 days. The green color fluorescent colonies were identified as Pseudomonas species and picked up for subsequent cultures on same media to obtain pure cultures. The identity of the bacteria as Pseudomonas was ascertained on the basis of Gram's Staining, Catalase enzyme activity, Urease activity and Fluorescent pigment formation [26], [27]. The cultures were regularly maintained in same medium at identical conditions and sub cultured at regular intervals.

3) Isolation and Characterization of Pure Culture of Lactobacillus species

Lactobacillus species were isolated from fresh curd samples [28]. Serial dilutions of 10-1 to 10-10 were made and poured on MRS Agar (peptone 10g, meat extract 10g, yeast extract 5g, D-glucose 20g, Tween-80 0.1g, K2HPO4 2g, sodium acetate 5g, tri ammonium citrate 2g, MgSO4.7H2O 0.2g, MnSO4.4H2O 0.05g, agar 1.5g and DDW to make up volume up to 1L; pH= 6.2-6.6) and incubated at 32ºC for 2-3 days in a B.O.D. incubator [29]. The colonies of Lactobacillus were isolated on the basis of colony shape (oval) and color (milky white). The isolates were repeatedly sub cultured and maintained on same medium to obtain a pure culture for the isolates. The further identification was done by similar assays as performed for Pseudomonas.

4) Culture of microbes for experimental setup

The cultures of Rhizopus spores, Pseudomonas and Lactobacillus were maintained per se and in combination on nutrient agar (peptone 10g, meat extract 10g, sodium chloride 5g ,1.5% agar, DDW to make up volume 1L; pH 7.2 ± 0.2) at 28ºC in a B.O.D. incubator for 24 hrs before inoculation for fermentation [29]. All media used for culture processes were prepared and autoclaved before use.

5) Experimental Set-up of fermentation for B12 synthesis

Fermentation set-up was done as per Liem et al [30]. Briefly, soybean, Bengal gram and rice grains were soaked overnight, de-hulled and boiled in DDW with 1% acetic acid for 30 mins. The grains were spread on an absorbent bed to drain off excess water before inoculation with culture microbes. The petriplates for fermentation were incubated with selected grain and microbial species combination at 28ºC, 85% humidity for 3 days in a B.O.D. incubator or till soybeans were covered with dense white mycelium. The combinations were set up as given in Tables 3-6.

6) Analysis of vitamin B2, B6 and B12 content

The fermented products obtained were pulverized at 104ºC for 10 minutes in a microwave oven and ground to fine powder. The powdered sample were dissolved in 0.1N HCl (3g/100ml) and analyzed by UV/visible spectrophotometer according to Özgür et al. [31]. Briefly, second derivative spectra were obtained with 0.1N HCl as reference and λmax267 for B2, λmax309 for B6 and λmax361 for B12. Second derivative calibration curves were obtained for standard vitamin B2, B6 and B12 solutions (each 3g/100ml) by using dilutions of stock solution (8-20µg/ml), each in the absence of the other.

3. Results

1) Characterizations of microbes

The cultures of fermenting microbes were isolated by using selective media and characterized as per stated methodology. The identity of Rhizopus was ascertained by cotton- blue lactophenol stained temporary preparation microscopy. The formation of white wooly mycelium was observed with the presence of root-like rhizoids at the base of sporangiophores. The characterization of bacterial species, Pseudomonas and Lactobacillus was done on the basis of Gram's staining, Urease test, fluorescent pigment formation, catalase test and H2S formation test. The results of the assays performed have been summarized as in Table 1.

Table 1
Characterization of Pseudomonas and Lactobacillus species

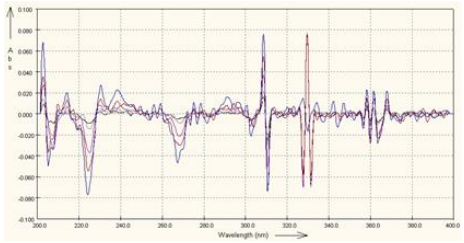
Assay Performed	***Pseudomonas***	***Lactobacillus***
Gram's Staining	-	+
Urease test	-	-
Fluorescent Pigment formation	+	-
Catalase test	+	-
H_2S formation test	+	+

(+) test positive, (-) test negative

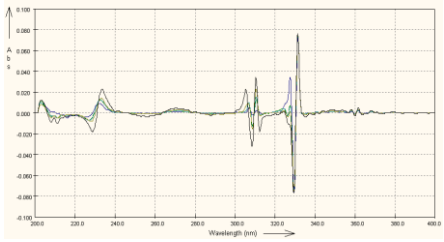
2) Calibration graphs for standards

The second order derivative spectra of standard vitamin B2, B6 and B12 were obtained as depicted in Figure 1 followed with regression analysis. Regression analysis was done for the slope, intercept and correlation coefficient values. The calibration equation was given by Y= ac+ b, where c is the concentration of the solution, Y is measured absorbance peak, a (slope) and b (intercept) are constants. The statistical data for calibration graphs has been summarized in Table 2. By substituting the corresponding experimental values, the calibration equation for second order derivative from Figure 1 was calculated as Y = 0.0027c + 0.0998 for B2, Y = 0.00088c + 0.0975 for B6 and Y = 0.24×10-3c + 0.64×10-3 for B12, which gives the best fit

straight line, that is the graph obtained for second order derivative spectra showed linear relationship.



Standard (B2)



Standard (B6)

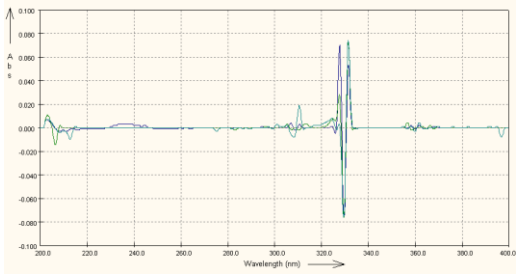
Table 2
Statistical data for Calibration graph

	Vitamin B_2 $^2D_{267}$	**Vitamin B_6** $^2D_{309}$	**Vitamin B_{12}** $^2D_{361}$
Correlation Coefficient (n=5)	0.895	0.980	0.9638
Slope (a)	0.0027	0.00088	0.24×10^{-3}
Intercept (b)	0.0304	0.975	0.64×10^{-3}

Standard (B12)

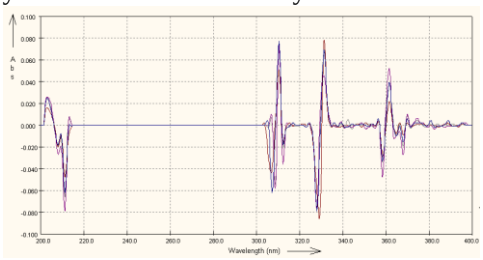
Fig. 1. Second order Derivative spectra of standard B2, B6 and B12 (8 - 20 µg/ml)

3) Vitamin content in different fermented samples

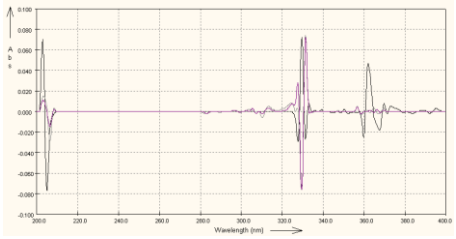


(a) Controls (without fermentation)

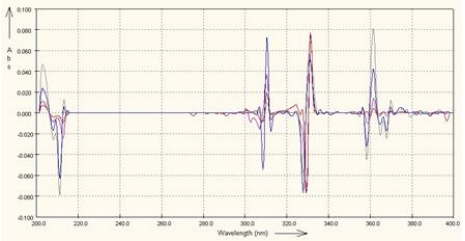
The quantification of vitamin B2, B6 and B12 as tabulated in Table 3 showed the presence of all the three in soybeans. The combinations of soybean with rice or soybean with Bengal gram did not show any difference in the quantity of vitamin B2, B6 or B12. It can be inferred that rice and Bengal gram do not contain any of these vitamins naturally.



b) Soyabean



(c) Soyabean+ Gram



(d) Soyabean + Rice

Fig. 2. Second Derivative Spectra of different Tempe samples.

a) Blue-Soybean, green- Soybean + gram, light blue-Soybean+ Rice;
b) Grey-Rhizopus, purple- Rhizopus+ Pseudomonas, red- Rhizopus +Lactobacillus, Blue – Rhizopus + Pseudomonas+ Lactobacillus;
c) Grey-Rhizopus, purple- Rhizopus+ Pseudomonas, red- Rhizopus +Lactobacillus, Blue - Rhizopus+ Pseudomonas+ Lactobacillus;
d) Purple – Rhizopus, blue- Rhizopus+ Pseudomonas, red- Rhizopus +Lactobacillus, Grey- Rhizopus+ Pseudomonas+ Lactobacillus.

Fermentation of soybean, as given in Table 4, by Rhizopus per se or in combination with Pseudomonas, Lactobacillus or both bacterial strains resulted in no change of B2 content. However, a decrease in vitamin B6 content was observed for all combinations. There was sharp rise in vitamin B12 quantity from 2.66 µg/ml× 10-3 in unfermented soybean to 87.33-210 µg/ml× 10-3for all combinations tested. The yield of B12 was least of all combinations where the fermentation was carried out in presence of Rhizopus and Lactobacillus.

Table 5 summarizes the results of fermentation of substrate combinations viz. soybean and Bengal gram. As was expected, no change in vitamin B2 content was observed in any of the combinations. There was overall decrease in vitamin B6 except in the case of fermentation with Rhizopus and Pseudomonas mixed culture. The content was equivalent to that obtained in natural soybean indicating no effect of fermentation was observed in this particular setup.

An increase in B12 quantity was observed for all combinations with minimum rise in combination with three strains and maximum in fermentation with Rhizopus+ Pseudomonas. The fermentation with Rhizopus per se and in combination with Lactobacillus yielded quantities intermediate of the two extremes. The results for experimental fermentation setup for soybean+ rice combination have been tabulated in Table 6. As is evident no change in vitamin B2 content was observed. The vitamin B6 content decreased in all combinations with maximum decrease in the group including all the three microbial species. B12 content was observed to rise as compared to unfermented soybean + rice combination; however the rise was relatively less in fermentation with Rhizopus+ Lactobacillus followed by Rhizopus alone. Maximum quantity was obtained when fermentation was accomplished by combination of all three experimental microbial species.

4. Discussion

The members of the genus Rhizopus have been vastly used as bioconversion organisms and include edible species of R. oligosporus and R. oryzae as used in fermentation of many South-East Asian foods [32], [33], [34]. The genus is characterised by broad range temperature (25-45ºC) and pH (4.5-7.5) tolerance, wide substrate dependency, lack of toxicity and ability to produce products of industrial importance [35]. Moreover they are known to enhance biosynthesis of B vitamins and antioxidants during fermentation process [36]. Thus, Rhizopus was the most likely candidate for the present

Table 3
Comparison of Vitamin B2, B6 and B12 quantity in non-fermented soybean samples as obtained by second order derivative spectrophotometric method followed by statistical analysis.

S.No.	Combination of Grains used	Combination of Microbes used	B_2 (µg/ml)	Change	B_6 (µg/ml)	Change	B_{12} (µg/ml×10^{-3})	Change
1	Soybean (control)	No microbe	36.963	↔	1107.95	↔	2.66	↔
2	Soybean+ Bengal Gram (1:1) (control)	No microbe	36.963	↔	1107.95	↔	2.66	↔
3	Soybean + Rice (1:1) (control)	No microbe	36.963	↔	1107.95	↔	2.66	↔

↔ No change, ↓ decrease, ↑ increase

Table 4
Comparison of Vitamin B2, B6 and B12 quantity in fermented soybean samples as obtained by second order derivative spectrophotometric method followed by statistical analysis.

S.No.	Combination of Grains used	Combination of Microbes used	B_2 (µg/ml)	Change	B_6 (µg/ml)	Change	B_{12} (µg/ml×10^{-3})	Change
1	Soybean	*Rhizopus*	36.963	↔	1027.04	↓	164	↑
2	Soybean	*Rhizopus+ Pseudomonas*	36.963	↔	1027.04	↓	210	↑
3	Soybean	*Rhizopus+ Lactobacillus*	36.963	↔	1052.5	↓	87.33	↑
4	Soybean	*Rhizopus+ Pseudomonas +Lactobacillus*	36.963	↔	1018.86	↓	164	↑

↔ No change, ↓ decrease, ↑ increase

Table 5
Comparison of Vitamin B_2, B_6 and B_{12} quantity in soybean + Bengal gram combination fermented samples as obtained by second order derivative spectrophotometric method followed by statistical analysis.

S.No.	Combination of Grains used	Combination of Microbes used	B_2 (µg/ml)	Change	B_6 (µg/ml)	Change	B_{12} (µg/ml×10^{-3})	Change
1	Soybean+ Bengal Gram (1:1)	*Rhizopus*	36.963	↔	1028.40	↓	289	↑
2	Soybean+ Bengal Gram (1:1)	*Rhizopus +Pseudomonas*	36.963	↔	1107.95	↔	330	↑
3	Soybean+ Bengal Gram (1:1)	*Rhizopus+ Lactobacillus*	36.963	↔	1079.77	↓	177	↑
4	Soybean+ Bengal Gram (1:1)	*Rhizopus+ Pseudomonas +Lactobacillus*	36.963	↔	1055.22	↓	80.66	↑

↔ No change, ↓ decrease, ↑ increase

Table 6
Comparison of Vitamin B2, B6 and B12 quantity in soybean+ Rice fermented samples as obtained by second order derivative spectrophotometric method followed by statistical analysis.

S.No.	Combination of Grains used	Combination of Microbes used	B_2 (µg/ml)	Change	B_6 (µg/ml)	Change	B_{12} (µg/ml×10^{-3})	Change
1	Soybean+ Rice (1:1)	*Rhizopus*	36.963	↔	1096.59	↓	50.66	↑
2	Soybean+ Rice (1:1)	*Rhizopus+ Pseudomonas*	36.963	↔	1070.68	↓	174	↑
3	Soybean+ Rice (1:1)	*Rhizopus+ Lactobacillus*	36.963	↔	1085.22	↓	10.66	↑
4	Soybean+ Rice (1:1)	*Rhizopus+ Pseudomonas +Lactobacillus*	36.963	↔	1027.04	↓	330.66	↑

↔ No decrease, ↑ change, ↓ increase

studies. The pure cultures of Rhizopus were isolated and purified from soy seeds sandwiched and incubated between Hibiscus leaves. Hibiscus was chosen as it is known to be naturally infected with various epiphytic fungal species, of which Cladosporium and Rhizopus are predominant [25]. Interestingly, the other epiphytic fungi are less competitive and do not interfere with pure culture isolation. These leaves serve as good attachment surface with adequate moisture retaining capacity and aeration for optimum fungal growth [25].

Aerobic P. denitrificans along with S. meliloti, B. megaterium and anaerobic S. typhimurium, Propionobacterium spp. have been known to produce B12 substantially [14], [37]. The species has particularly proved useful because of their rapid growth, genetic accessibility, high yields and less stringent nutrient requirements [38]. Pseudomonads can possibly thrive on different carbon and nitrogen sources and thus culturing and maintaining them is relatively easy on industrial scale. Their widespread ecological distribution and capability to produce a diverse range of industrial products have made them organism of choice for commercial application and henceforth for the present studies too. The isolation of Pseudomonas was done from nodulated plant roots as they are home to a number of nitrogen fixing microbes, with evidences for some of them as vitamin B-complex producers [14]. Thus, the nodulated roots were ground in a pestle and mortar to obtain a suspension of these microbial strains. The serial dilutions of this suspension followed by pouring on selective media (Pseudomonas Agar) helped to obtain pure cultures of Pseudomonas strains. The identity of genus was in conformation with similar reports [39], [40].

The Lactobacilli strains have found to be suitable for development of functional foods which may be enriched with important dietary metabolites. Many recent studies have identified new Lactobacilli species that can produce vitamin B complex especially B12 following fermentative procedures [41], [42], [43], [44]. Taranto et al were first to report B12 production in Lactobacilli species [45]. Therefore; Lactobacillus was third group of choice for these studies. The isolation of Lactobacillus strains was preferably done from domestic curd as it is a natural source of Lactobacillus strains [28], [46], [47]. The isolation and purification was carried out on selective media (MRS Agar) as per procedures followed for Pseudomonas [29]. The assays performed as per Table 1 established the presence of genus Lactobacillus in the isolates obtained from curd. A reference to Bergey's manual confirmed the identity of the genus Lactobacillus [48].

The bacteria-fungi interactions have been largely explored in mass production of fermented foods and beverages likely due to altered production of secondary metabolites [49]. The fermentation by these microbes results in metabolism or degradation of complex compounds to yield nutrient rich compounds [50]. Besides, these interactions improve food quality, texture, sensory attributes and shelf life of fermented foods.51 Thus, it was relevant to observe effects of these interactions on B12 production in these studies which incorporated Rhizopus as the essential fermentative microbe and Pseudomonas, Lactobacillus or both as additional microbes for mixed cultures.

The growth requirements of different microbial species depend on a host of factors and nutrition being one of them is most critical. The quantity and constitution of nutrients may vary with species, some nutrients may be essential and others may be non-essential. Some of the microbes metabolize simple sugars from nutrient substrates more readily as compared to complex starches, celluloses or glycogen available in foods of plant and animal origin respectively. The alternative may be true for other classes of microbes. Still, some microbes may be better metabolizers of fats and amino acids for their nutrient requirements [52]. On the contrary, some microbes are unable to synthesize nutrients necessary for growth and thus depend on nutrients produced by other organisms. Such interactions have been crucial in food processing industry for development of fermented food products [49]. This ability of microbes to process raw substrates and convert them as nutrient substrate for another group of microbes, which in turn synthesize products of commercial application aroused an interest to experiment with combinations of soybean, Bengal gram or rice. Soybean is naturally found to have high protein content. Both Bengal gram and rice are diverse in nutrient composition, Bengal gram being rich in proteins and rice being rich in sugars like starch. Thus, an experimental set up of soybean and Bengal gram provided protein rich substrate with meager content of sugars. In contrast, the soybean plus rice substrate can be assumed to be proportionate as soybean and rice furnish protein and carbohydrate requirements respectively. The effect of these combinations and mutual interaction of microbes are clearly evident from results summarized in Tables 3-6 and as discussed henceforth.

The general methods available for assay of B vitamins in food sources include microbiological assays, direct spectrophotometry, Inductive-coupled Plasma-Mass Spectrometry (ICP-MS), Atomic Absorption spectroscopy, HPLC and Capillary electrophoresis [53], [54], [55], [56], [57]. The microbiological assays are simple, but tedious and time consuming. Further, these are not sensitive and reliable and hence have become obsolete. Direct spectrophotometry is also simple and inexpensive technique but cannot be relied on for complex mixture having components with overlapping spectra, limiting its scope and application. The other methods available are expensive and skill intensive. Their use and interpretation requires training and experience, a high institutional set up cost which limits its reach to most of the educational institutions in developing nations. All the above reasons push for a need to find an analytical method that could reasonably resolve the issues of skill and cost. Derivative spectrophotometry has become the method of choice for researchers, at beginner's level, who can now conveniently identify each component of complex mixtures [58], [59], [60]. It is an analytical technique which can qualitatively as well as quantitatively resolve the unresolved bands in spectra. The errors due to baseline shifts and tilts, medium turbidity, complex nature of mixtures, matrix background and light refraction are simultaneously eliminated [58]. The method is cost effective and has been validated for pharmaceutical formulations [31], [59], [61]. Moreover, it is

simple, single step method (no extraction of vitamins) for vitamin B assay in food samples with no effect of interference from other molecules.

The present study uses second order derivative spectrophotometry to resolve B complex vitamins viz. B2, B6, B12 which have spectral peaks close to each other and hence cannot be distinctly identified in a complex mixture like food samples. Fermented products obtained from soybean per se or in combination with similar grains contains both B2 and B6, making them good source for diet supplements. Thus, an analysis of B2 and B6 was also done in addition to B12 analysis. The B2 content as given in Tables 3-6 and Figures 2 (a-d) remained constant for all fermented samples obtained. This indicates that neither B2 was lost during experimental procedures nor it was being produced by any of the experimental microbes.

The derivative spectra of all experimental samples as in Figures 2 (a-d) exhibited reduction of B6 content following fermentation as compared to control unfermented grains. The quantification by calibration equation also suggests similar observations as summarized in Tables 3-6. The reduction in B6 content may be due to uptake solely by Rhizopus sp. to fulfill its own nutritional requirements for growth [62]. In addition to above observation it is also suggested none of the experimental microbes produced B6 during fermentation, thus no increase in its content was noticed. A deviation, from the otherwise generalized observations, was observed when Rhizopus was cultured in presence of Pseudomonas on soybean + Bengal gram substrate (Table 5) may be due to capacity of Pseudomonas to produce vitamin B6 [63]. The second order derivative spectra, given in Figures 2 (a-d) established the presence of B12 in soybean in accordance to some earlier reports [64], [65]. However, B12 was not found in either Bengal gram or rice and the B12 content found in combinations may have been contributed by soybean alone. The fermentation of soybean with Rhizopus per se and in combination with Pseudomonas, Lactobacillus resulted in increase of B12 as evident in Figure 2b. It was surprising to find B12 in samples fermented with Rhizopus only as it known to not produce B12. The B12 may have been contributed from steps performed before fermentation was started [66]. The increase in B12 quantity was highest for Rhizopus + Pseudomonas combination as Pseudomonas is a known B12 producer [67], [68], [69]. Lactobacillus strains have been traditionally used as experimental microbes in microbiological assays for vitamin B12 as these metabolise B12 rather than produce it [70], [71].

The fermentation on soybean + Bengal gram substrate had higher quantity of B12 when same combination of microbes were used, however, the content was higher than that for substrate containing soybean only, as observed from Table 5. The high protein content of both soybean and Bengal gram may have promoted better growth of microbes and thus enhanced B12 synthesis. The observations for soybean + rice combination substrate (Figure. 2d) were also similar to other samples. The increase was marginal in case of fermentation with Rhizopus + Lactobacillus as compared to other fermented substrate which may be because Lactobacillus species are generally known not to produce B12. The increase in B12 for this group of combinations was highest in Rhizopus+ Pseudomonas+ Lactobacillus, Table 6, due to the nature of carbohydrate content of rice being easily available for metabolism by microbes. The favorable carbohydrate source viz. starch, glucose, sucrose etc. from rice in contrast to soybean or Bengal gram causes increase in population of B12 producing Pseudomonas resulting in enrichment of this fermented sample with B12. Wolkers–Rooijackers et al have also reported similar increment in B12 content in a co-culture of Rhizopus with Propionibacterium [71].

5. Conclusion

Second order derivative spectrophotometry was applied as a method of choice to detect and quantify the B complex vitamins viz.B2, B6 and B12. The method proved to be easy, reliable, sensitive and cost effective in detecting these vitamins distinctly, though present in traces (in µg). It was observed the expression of vitamin B complex content can be affected as per the choice of combinations of microbes and grains. In the present study it was found that Rhizopus+ Pseudomonas +Lactobacillus combination was best microbial combination that can be used for fermentation dependent B12 synthesis. The grain combination of Soybean+ Bengal Gram was best when compared to Soybean alone or Soybean+ Rice as B12 yield increased in all samples irrespective of the microbial combination.

In this work the effect of substrate on B12 synthesis by three microbial species Rhizopus, Lactobacillus and Pseudomonas was studied. The results show a possibility of increased B12 concentration by adopting multiple combinations of substrate in presence of variable microbial inoculates. The obtained results emphasise on selection of an appropriate raw material for B12 synthesis in large quantities. Further studies are required for scaling up these results to industrial applications to meet the rising demand in chemical and pharmaceutical industry. Studies may also be undertaken to explore the possibility of exploiting microbial interactions in improving food nutrients like vitamin B12, that are limited to animal origin foods.

6. Acknowledgement

The author acknowledges and appreciates the contribution of two students Ms Upasana Pandey and Ms Shivangi Singh who were pursuing their Masters and assisted with laboratory procedures and data collection.

Declarations- Author declares there is no conflict of interest.

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