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Microbiological Studies on the Production of Vitamin B_{12} from Two Mixed Cultures under Solid State Fermentation Condition

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Abstract: Five types of agricultural crop residues were used in the present study as a source of biotechnological application namely sugar-cane bagasse, wheat straw, rice straw, bean straw and cotton stalks. A total of 140 microbial isolates, 107 bacteria (76.42 %) and 33 actinomycetes (23.6 %) were isolated from five different soil samples (sandy loam, clay loam, sandy clay loam, sandy calcareous and sandy soils). Only two microbial isolates one bacteria isolate (well identified as *Bacillus firmus* AZ-78B) and one actinomycete isolate (well identified as *Streptomyces halstedii*, AZ- 8A) were found to produce significantly higher yield of the vitamin B_{12} (37.7µg/ml). Determination of vitamin B_{12} production was carried out using *E.coli*, ATCC 14169 as the test organism. The parameters controlling the biosynthetic process of vitamin B_{12} formation including different pH values, different temperatures, deferent incubation period, and deferent carbon and nitrogen sources and different mineral salts concentrations were fully investigates. The fermentation broth was extracted by using n-Butanol. Purification of the vitamin B_{12} was performed by using column chromatography technique. Precipitation of the vitamin B_{12} up to crystalloid form was fully investigates. The spectroscopic analysis (UV, IR and HPLC spectrum) were used for comparative studies between purified compound produced by mixed cultures and standard vitamin B_{12}. Recorded data emphasized the fact that, the purified compound was suggestive of being belonging to vitamin B_{12}.

Key words: Vitamin B_{12}; *Streptomyces* sp.; *Bacillus* sp. *Escherichia coli*, ATCC-14169. Parameters controlling on the biosynthesis vitamin B_{12}; Production, Extraction and Purification; Crystallization of *vitamin* B_{12}; Spectroscopic analysis and Solid state fermentation condition.

INTRODUCTION

Solid state fermentation (SSF) and submerged fermentation (SMF) were used for centuries and are still used today as principal technology for agricultural wastes to produce biological best control agents using microorganism's cultivation on moist solid raw materials, such as corn stalks, cotton stalks and sugar–cane bagasse. This is an alternative to cultivate microorganisms in liquid nutrients broth[5].

Cyanocobalamin is a vitamin commonly known as vitamin B_{12}. The name vitamin B_{12} is used in two different ways. In a broad sense it refers to a group of cobalt-containing compounds known as cobalamins-cyanocobalamin (an artifact formed as a result of the use of cyanide in the purification procedures), hydroxocobalamin and the two coenzyme forms of B_{12}, methylcobalamin (MeB_{12}) and 5-deoxyaden-osylcobalamin (adenosylcobalamin ($AdoB_{12}$)[3]. In a more specific way, the term B_{12} is used to refer to only one of these forms, cyanocobalamin, which is the principal B_{12} form used for foods and in nutritional supplements[42]. Vitamin B_{12} (Cyanocobalamin) is a red crystalline cobalt complex synthesized by microorganisms. Khan and Easwaran[20].

reported that the formula for vitamin B_{12} is $C_{63}\ H_{88}\ O_{14}\ N_{14}\ PCo$. The central cobalt atom is linked to four reduced pyrrol rings, forming a macro ring. Three of four junctions between the rings take the form of the *meso* or bridge carbon atom characteristic of the porphyrins. In the fourth piece, however, there is a direct linkage between the two α-carbons of rings "D" and "A". The macro ring almost certainly contains six conjugated double bonds constituting a unique resonating system[22].

The third hydroxyl function of the phosphate group was though to be also esterified, until it becomes clear that instability of triesters of phosphoric acid precluded such as Vitamin B_{12} is, in fact an inner salt; the negative charge on the phosphorous atom is neutralized by a positive charge on the cobalt on the co-ordination complex[38]. Cobalamin is involved as a cofactor in

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the transfer of methyl groups. It is needed to remove the methyl group from methyl tetrahydrofolate so that tetrahydrofolate can be used for the synthesis of DNA. In the absence of vitamin B_{12}, DNA is not produced and the cells grow without dividing, becoming megaloblasts[21]. Vitamin B_{12} plays a key role in nerve cell activity and with folic acid regulates homocysteine levels[6].

In the present work, we describe only two microbial isolates (well identified as *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A), were found to produce significantly higher yield of the vitamin B_{12} and studies the factors effecting on the biosynthesis process of vitamin B_{12}. The vitamin B_{12} was extraction, precipitation and purification, and it's the spectroscopic analysis (UV, IR and HPLC spectrum) have been investigated.

MATERIALS AND METHODS

Mixed Cultures Used for Biosynthesis of Vitamin B_{12}: The two mixed cultures were isolated from wheat sandy clay loam sample for actinomycete isolate and sandy clay loam cotton stalks for bacterial isolate as agricultural waste and well identified as *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A. The medium used for microbial isolates contained 20 gm of agricultural wastes supplemented with the mineral salts of modified Dox medium which consisted of (g/L) $NaNO_3$, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and agar 20. The mineral salts were dissolved and completed up to one liter by tap water and then autoclaved at 1.5 atm. for 30 min[2].

Test Organism Used for Determination of Vitamin B_{12}: Determination of vitamin B_{12} production in fermented extract was carried out using *Escherichia coli,* ATCC-14169.

Growth Medium: The medium used for microbial growth under solid state fermentation conditions (S.S.F) contained 3 gm of agricultural wastes supplemented with 30 ml mineral salts of Dox medium at neutral conditions and mineral salts of sato medium at alkaline conditions, After incubation, 50 ml of sterilized distilled water were added and shaken.

Assay media of vitamin B_{12}[24]:

Medium (A): It contained (g/500 ml): Potassium dihydrogen phosphate, 3.0; Dipotassium hydrogen phosphate, 7.0; Trisodium citrate. $2H_2O$, 0.5; Magnesium sulphate. $7H_2O$, 0.1; Ammonium sulphate, 1.0; Tetrazolium hydrochloride, 0.2; and Distilled water up to 500 ml

Medium (B): It contained (g/500 ml): Agar-agar, 20.0; Glucose solution (30% w/v), 6.66 ml and Distilled water up to, 500 ml.

Fermentation Growth Media: The medium used for production of vitamin B_{12} under solid state fermentation conditions (S.S.F) contained 3 gm of agricultural wastes supplemented with 30 ml mineral salts of following basal medium. It contains (g/l) Ammonium chloride 2.0 g; KH_2PO_4 1.0 g; $MgSO_4.7H_2O$ 0.5 g; KCl 0.5 g; Yeast extract 2.0 g; Calcium carbonate 2.0 g; NaCl 3.0 g; Glycerol 5.0 ml; MnSO4 $.7H_2O$ 0.2 g; $CoCL_2.6\ H_2O$ 0.01 g; $FeSO_4.7H_2O$ 0.015 g; $ZnSO_4.7H_2O$ 0.02 g; $Na_2MO.2H_2O$ 0.005 g and Distilled water up to 1000 ml.

Screening of Vitamin B_{12} Productivity:

Qualitative Determination of Vitamin B_{12} Biosynthesis by Mixed Cultures[24]: To examine Vitamin B_{12} biosynthesis, each microbial isolates was grown on fermentation medium at 35 ^{0}C for 4 days. After incubation , and in order to convert the vitamin B_{12} analogues to cyanocobalamin a mixture of 10 ml of the fermentation medium and 1 ml of 0.2 M acetate buffer pH 5.5 containing 0.001% potassium cyanide was boiled in a water bath for 20 minutes. Thereafter, filter paper discs (Watman No. 3) were loaded with 20 ul of the resultant extract. Impregnated discs were then placed onto the surface of agar plates of assay medium seeded with *Escherichia coli*, ATCC-14169. After incubation at 37 °C for 18 hours, the diameters of exhibition zones were measured and taken as a rapid qualitative method of indication of vitamin B_{12} productivity.

Quantitive determination of Vitamin B_{12} productivity (Agar diffusion assay)[10,14,7] A mixture of 10 ml fermentation medium, after the end of the fermentation process and one ml of 0.2 M acetate buffer pH5.5 containing 0.001% potassium cyanide, was boiled in a water bath for 20 minutes. The undiluted mixture and a 50 times dilution were used for vitamin B_{12} assay. Determination of vitamin B_{12} was carried out using *E.coli*, ATCC 14169[10] as the test organism. The cells were separated from 18 hours old incubated culture of *E .coli*, ATCC 14169 and inoculated on nutrient agar slants by sterile saline solution. After centrifugation at 3500 r.p.m for 10 min, the cells were washed with sterile distilled water. The preceding process was repeated five times. The inoculum was diluted to give a 25% Transmission at 420 nm. The assay was performed on 30X30 cm glass plates 6 mm thickness. A base layer of 170 ml assay medium (A) and 130 ml medium (B) were mixed and then, 0.9 ml aliquot of the prepared inoculum suspension of *E .coli*, ATCC 14169 was added to the above mixture at 45°C. The inoculated medium was then poured on the large plate base layer. It was left undisturbed to solidify. The solidified medium was kept at 4°C. A total of 64 cups were bored. Then, samples (undiluted and 50

times diluted) and control (0.2 and 0.02 ug/ml authentic of vitamin B_{12}) were used to fill the cups using 8X8 Quasi-Latin Design distribution according to[14,24].

Parameter Controlling on the Biosynthesis of Vitamin B_{12}: These included incubation period, pH values, incubation temperatures; different carbon and nitrogen sources and different mineral salts concentrations have been determine by the standard methods.

Fermentation, Extraction and Purification of Vitamin B_{12}:

Fermentation: Six discs from each microbial growth (*Bacillus firmus* and *Streptomyces halstedii*) were introduced aseptically into each sterile 250 ml Erlenmeyer flask containing 5 g dry weight of mixed agricultural wastes supplied with 30 ml mineral salt solution at optimum conditions for maximum vitamin production. The pH was adjusted at 10 and incubated at 35 ^{0}C for 96 hrs. After incubation, 50 ml of sterilized distilled water were added and shaken. The fermentation medium was tested qualitatively for the presence of vitamin B_{12} according to method described in microbiological assay. *Escherichia coli*, ATCC 14169, used in assay medium was incubated at 37°C for 18 hours[36,16]. Highest yield, as indicated by the largest zone of exhibition, was then determined by large plate microbiological assay 8X8 Latin Square Design[14].

Conversion of Cobalamin to Cyanocobalamin in the Cultured Fermentation Medium: The suggested modified method to convert of cobalamin to cyanocobalamin was that of Takeuchi[39]. Two liters of the obtained fermented medium were prepared and pH was adjusted at 5.5. Potassium cyanide was added gradually to make a final concentration of 0.001% (w/w).

Extraction of Vitamin B_{12}: Fermented medium was adjusted at pH 5.5 and extraction process was carried out using different organic solvents to be added to fermentation broth at a level of 1:1 (v/v) respectively. The organic phase was collected, evaporated under reduced pressure by using a rotary evaporator.

Crystallization of Vitamin B_{12}: Evaporation was conducted until viscous syrup was obtained. The obtained residual extract of vitamin B_{12} was dissolved in 20 ml distilled water, and then treated with 100 g of activated carbon. After washing the activated carbon with water (120ml), the adsorbed matter by the activated carbon was eluted with 75% aqueous acetone solution (100 ml)[39]. Eluted matter was condensed under reduced pressure to two ml then passed through column chromatography[41].

Purification of Vitamin B_{12}:

Purification of Vitamin B_{12} by Column Chromatography Technique: A column of (2.5 X 50) Cm packed with silica gel (Prolabo) was used for this purpose. Although methanol: water: acetic acid (50:50:1 v/v)[24] were used as eluting solvent, a glass rod was often used to stir the slurry. Once the slurry get homogenous, it was poured cautiously into the empty column and left for over night until the silica gel was completely settled. One ml crude Vitamin B_{12} extract was added onto top of the silica surface. The eluting mixture reservoir was connected to the column. Fifty fractions were collected (each of 5 ml). Vitamin B_{12} assay was performed for each separate fraction.

Spectroscopic Analysis: The IR, UV and HPLC spectrum were determined at the micro analytical center of Cairo University, Egypt.

Acetate Buffer pH 5.5:

- A- 11.55 g Glacial acetic acid / liter= 0.2 M
- B- 16.4 g Sodium acetate/ liter= 0.2 M 4.8 ml of (A) and 45.2 ml (B) were mixed and completed up to 100 ml with distilled water.

RESULTS AND DISCUSSION

Screening for Vitamin B_{12} Productivity:

Qualitative Determination of Vitamin B_{12} Production by Mixed Cultures: Only two mixed cultures from two strains *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A were found to produce significantly higher yield of the vitamin B_{12}.were screened for vitamin B_{12} biosynthesis by growing on medium used for microbial growth under solid state fermentation conditions (S.S.F) contained 3 gm of agricultural wastes supplemented with the mineral salts.

Quantitive Determination of Vitamin B_{12} Productivity (Agar Diffusion Assay): Determination of vitamin B_{12} production in fermented extract was carried out using *Escherichia coli*, ATCC-14169 as test organism. Results recorded in Table (1), showed that two mixed cultures of (*Bacillus firmus*, AZ-78B and *Streptomyces halstedii*, AZ- 8A) gave the highest vitamin B_{12} productivity 37.7 µg/ml.

Parameter Controlling on the Production of Vitamin B_{12}1- Different pH Values: The maximum biosynthesis of vitamin B_{12} recorded at pH 10 by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* fig. (1)

Table 1: Quantitative determination of vitamin B_{12} production (mm) using 8x8 Quasi-Latin Design

	1		2		3		4		5		6		7		8	
	Fraction 4		Fraction 5		Fraction 6		Fraction 7		Fraction 8		Fraction 9		Fraction 10		*Standard	
	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H 0.2	L 0.02
	25.8	20.0	29.0	22.0	30.5	23.1	37.1	23.5	30.0		26.0	19.0	24.3	18.0	24.5	20.0
	25.5	19.5	29.6	21.9	30.8	22.8	37.7	23.0	29.5	21.0	26.0	19.0	24.0	18.1	24.0	20.3
	25.3	19.6	29.5	21.5	30.5	22.8	37.5	23.0	29.0	21.6	25.8	18.5	24.0	17.8	23.9	19.8
	25.5	19.3	29.0	21.6	30.7	23.0	37.9	23.4	29.7	21.6	25.8	18.8	23.5	17.5	24.0	20.0
	102.1	78.4	117.1	87	122.5	91.7	150.2	92.9	118.2	85.7	103.6	75	95.8	71.4	96.4	80.1
	Y_1	X_1	Y_2	X_2	Y_3	X_3	Y_4	X_4	Y_5	X_5	Y_6	X_6	Y_7	X_7	Y_8	X_8
ΣY	905.9															
ΣX	662.2															
Z	180. 5		204.1		214.2		243.1		203.9		198.6		167.2		176.5	
R	60.9															
D	4		27.6		37.7		66.6		27.4		22.1		-9.3			
M	0.066		0.45		0.62		1.1		0.45		0.36		-0.15			
Antilog M	1.16		2.81		4.17		12.5		2.8		2.3		0.7			
I	0.23		0.56		0.83		2.5		0.56		0.47		0.14			
p Mg/ml	11.6		28.1		41.7		125		28		23		7			
	Average concentration p / 7 = 37.7 µg / ml vitamin B12															

*standard (ug/ml); H= High dose (y) (undiluted); L= Low dose (x) (50-time diluted); ΣY= Total reading for the high dose; ΣX= Total reading for the low dose; Z= Total readings of low and high doses, R=Z/4 D= (Total of low and high dose of test) – (Total of low and high dose of standard), M=D/R, S= antilog M, I= Potency of the high dose test= SX 0.2 and P= Sample Potency= 1X50 (mean values= 37.7) .

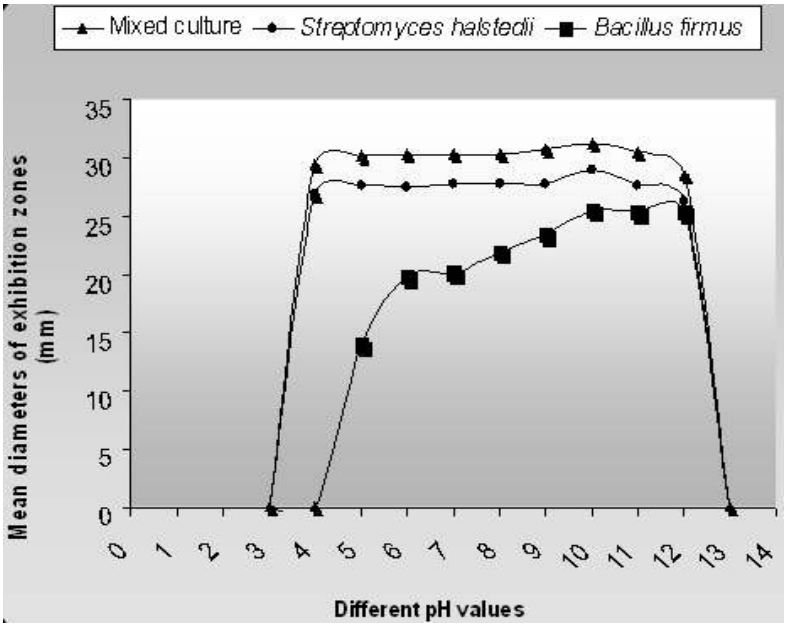


Fig. 1: Effect of different pH values on the production of vitamin B_{12} by the two microbial isolates under S.S.F. conditions.

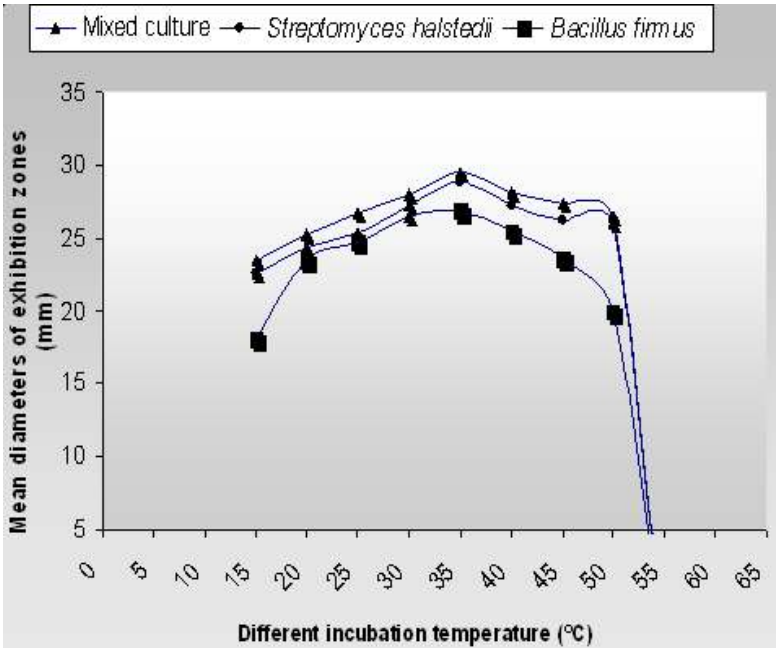


Fig. 2: Effect of different incubation temperature on the production of vitamin B_{12} by the two microbial isolates under S.S.F. conditions.

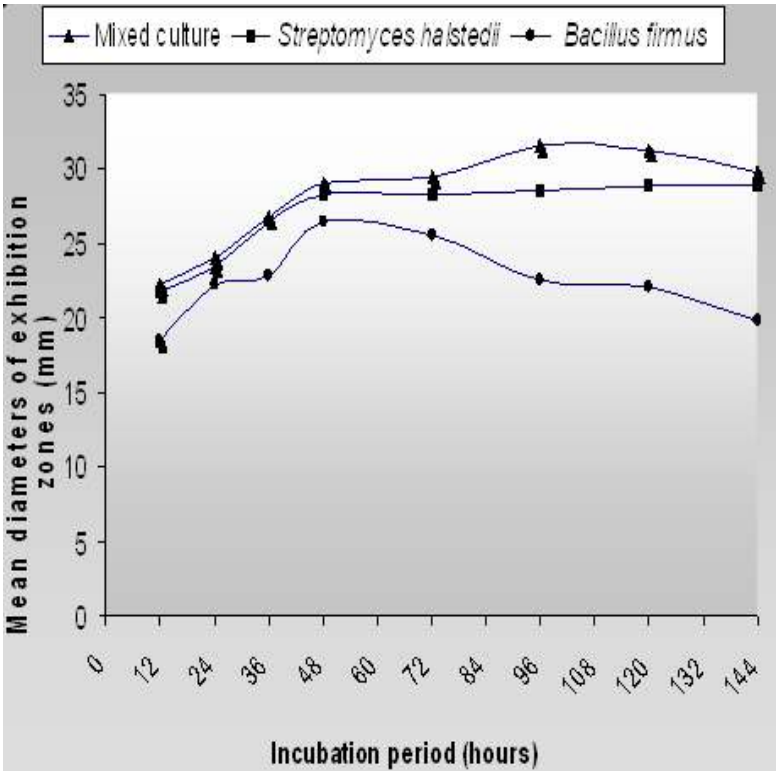


Fig. 3: Effect of different incubation periods on the production of vitamin B_{12} by the two selected microbial isolates under S.S.F. conditions.

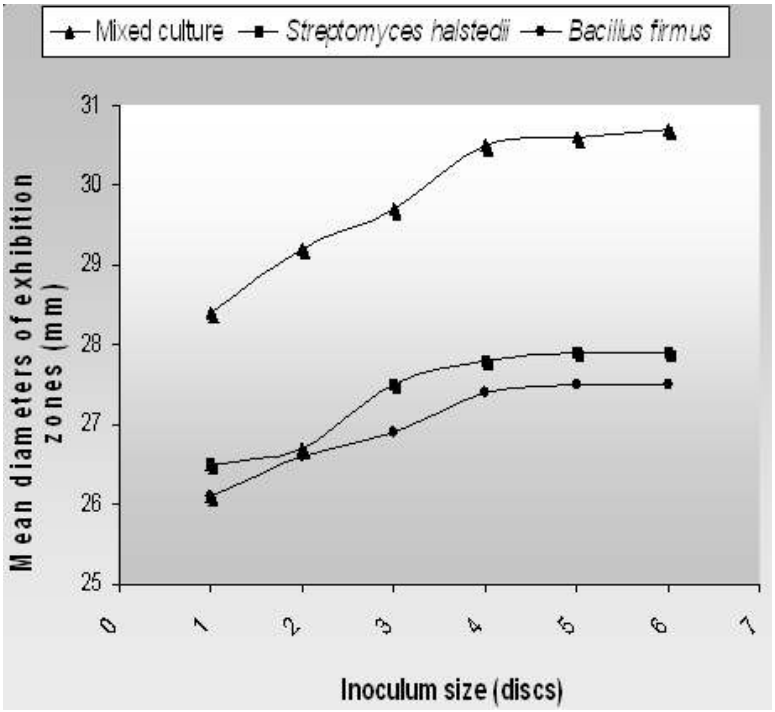


Fig. 4: Effect of the inoculum size on the production of vitamin B_{12} by the two microbial isolates under S.S.F. conditions.

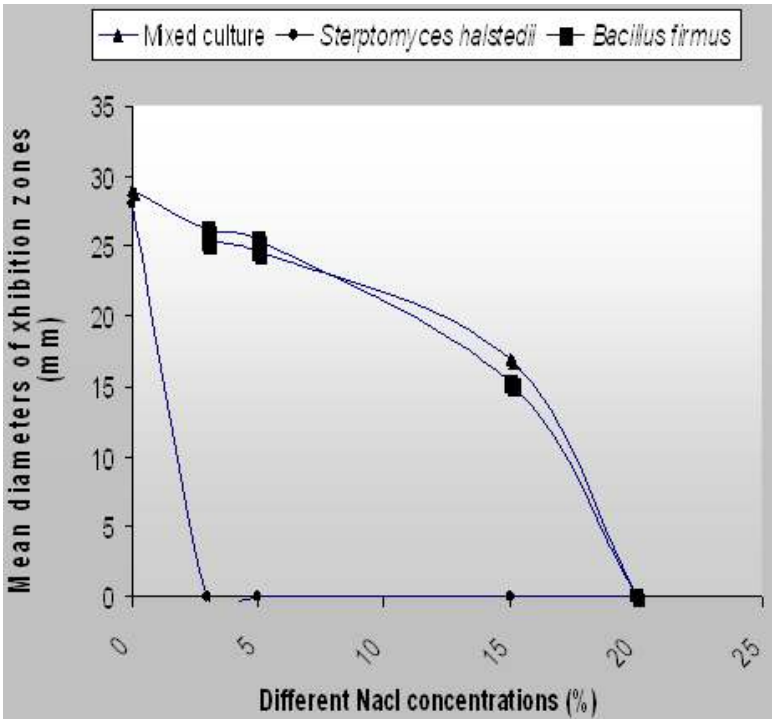


Fig. 5: Effect of different sodium chloride concentrations on the production of vitamin B_{12} by the selected microbial isolates under S.S.F. conditions.

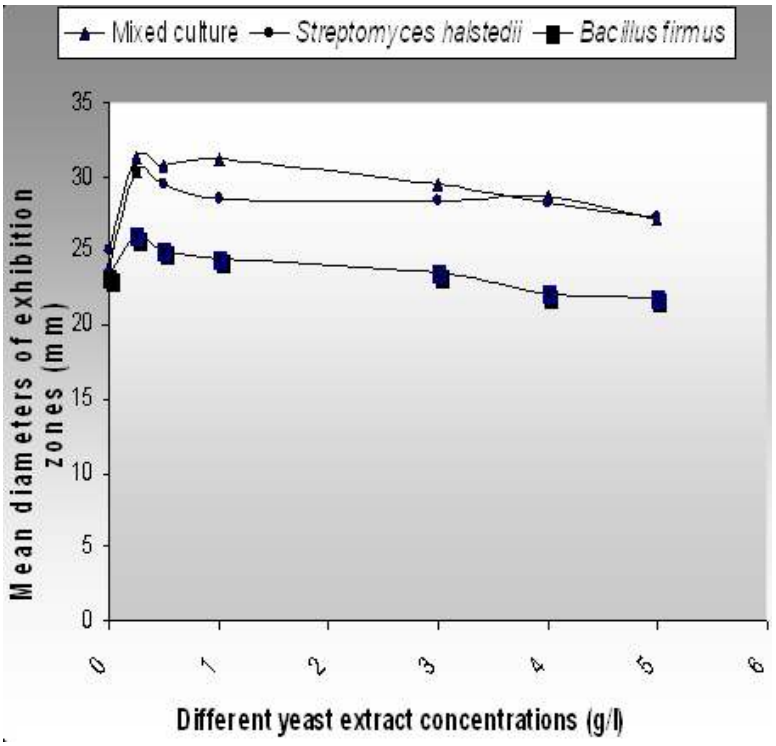


Fig. 6: Effect of different yeast extract concentrations on the production of vitamin B_{12} by the selected microbial isolates under S.S.F. conditions.

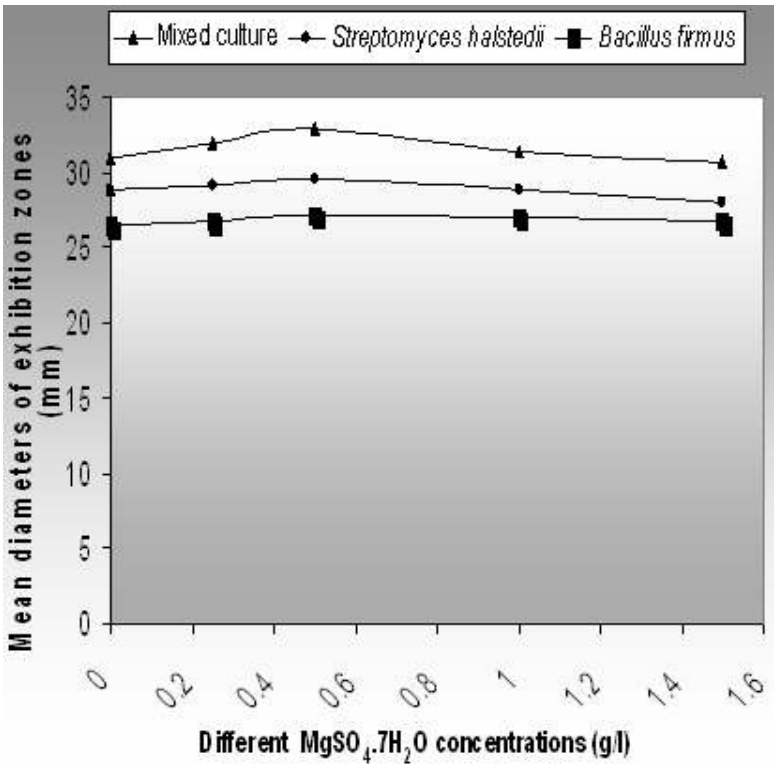


Fig. 7: Effect of different $MgSO_4.7H_2O$ concentrations on the production of vitamin B_{12} by the selected microbial isolates under S.S.F. conditions.

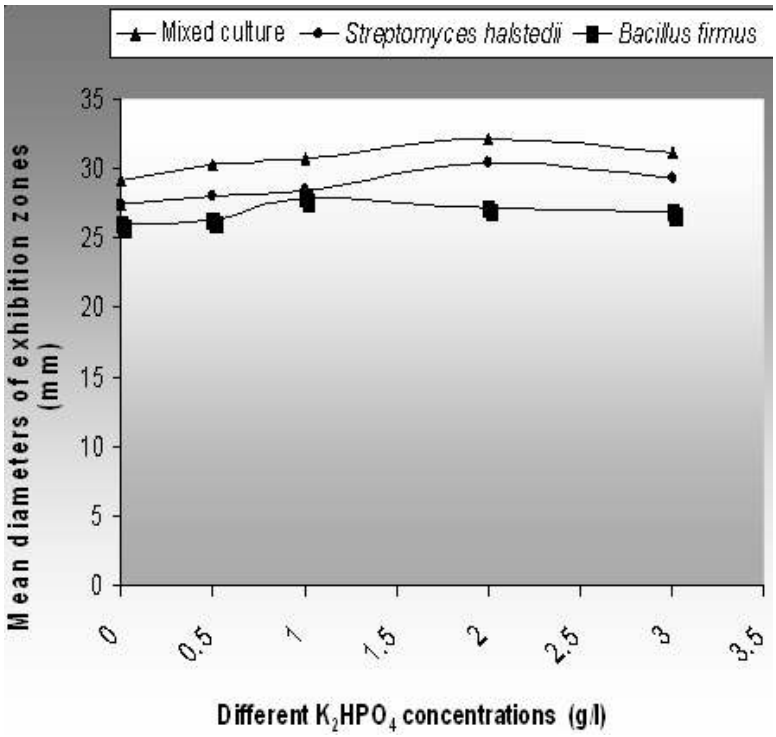


Fig. 8: Effect of different K_2HPO_4 concentrations on the production of vitamin B_{12} by the selected microbial isolates under S.S.F. conditions

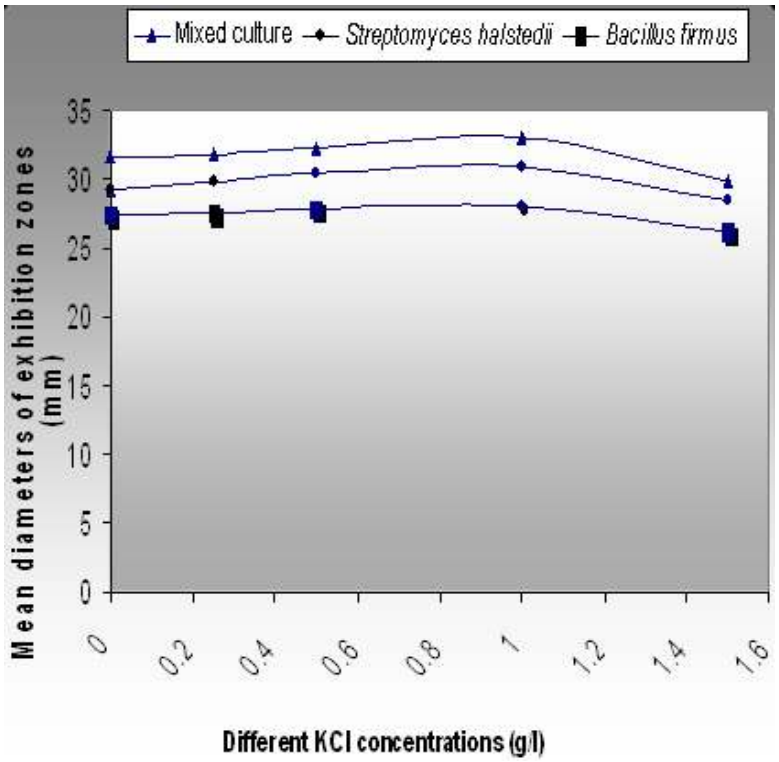


Fig. 9: Effect of different KCl concentrations on the production of vitamin B_{12} produced by AZ-78B & AZ-8A and mixed culture (AZ-78B & AZ-8A)under S.S.F.

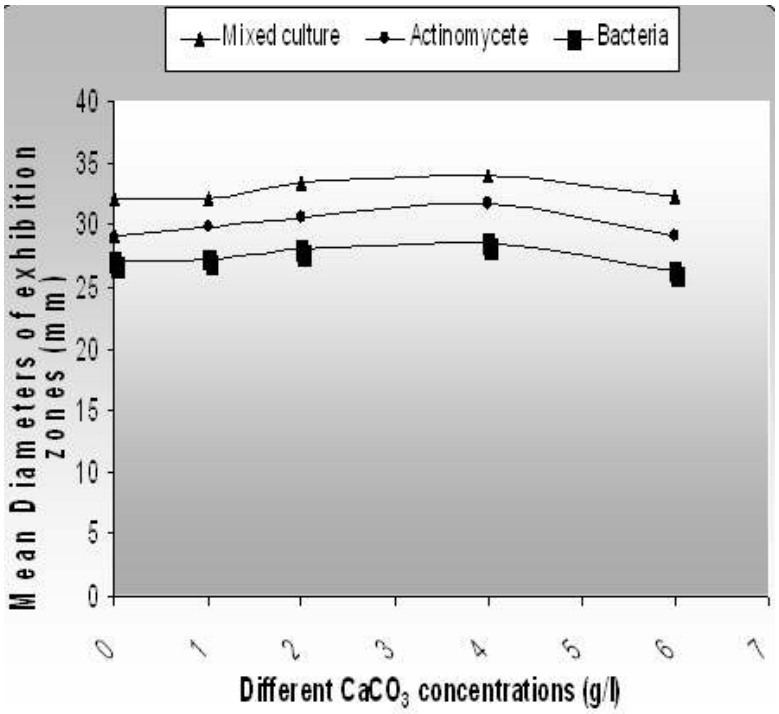


Fig. 10: Effect of different $CaCO_3$ concentrations on the production of vitamin B_{12} produced by AZ-78B & AZ-8A and mixed culture (AZ-78B & AZ-8A) under S.S.F.

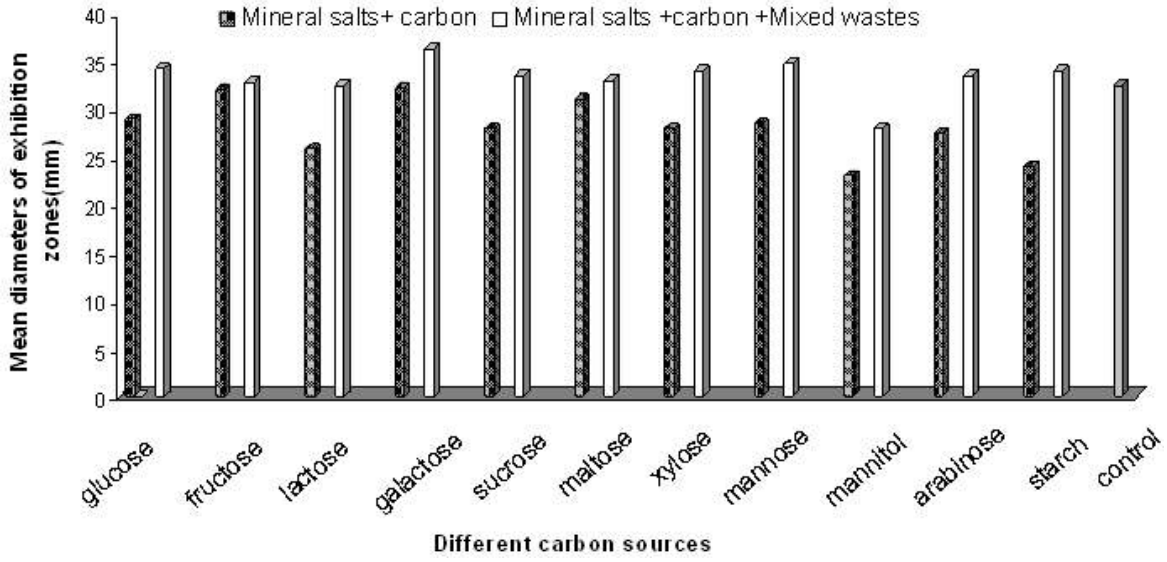


Fig. 11: The effect of different carbon sources on the production of vitamin B_{12} by AZ-78B&AZ-8A under S.S.F.

Different Incubation Temperature: The maximum biosynthesis of vitamin B_{12} could be recorded within an incubation temperature of 35°°C for mixed cultures of *Streptomyces halstedii* and *Bacillus firmus* fig. (2).

Different Incubation Period: The maximum biosynthesis of vitamin B_{12} was recorded with an incubation period 96 hrs. for mixed cultures of *Streptomyces halstedii* and *Bacillus firmus* (fig. 3).

Different Inoculum Size: The maximum vitamin B_{12} biosynthesis was obtained in the presence of 6 discs by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 4).

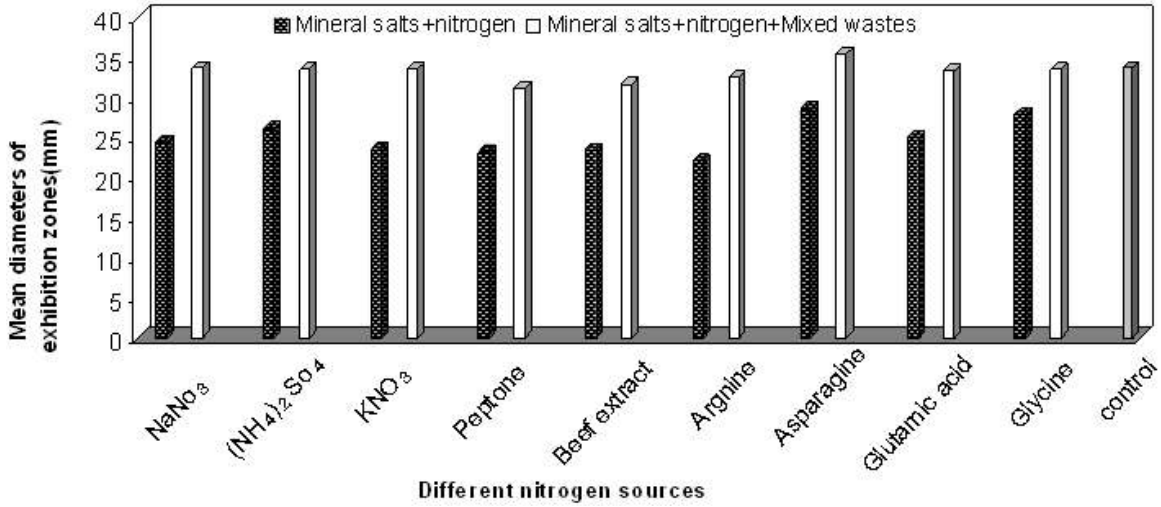


Fig. 12: The effect of different nitrogen sources on the production of vitamin B_{12} by AZ-78B&AZ-8A under S.S.F.

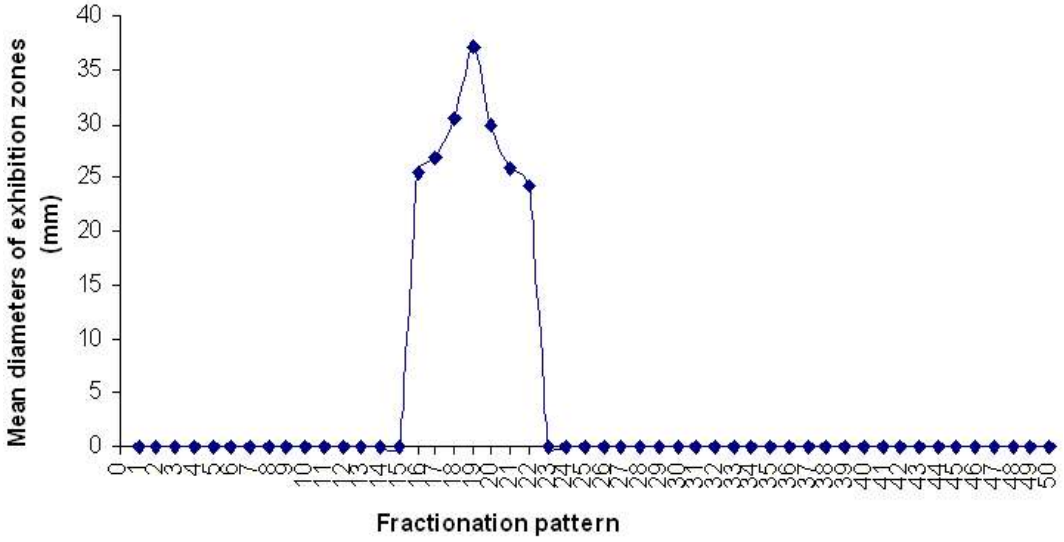


Fig. 13: Promoting effect of fractionation pattern of vitamin B_{12} produced by AZ-78B&AZ-8A under S.S.F. using silica gel column chromatography.

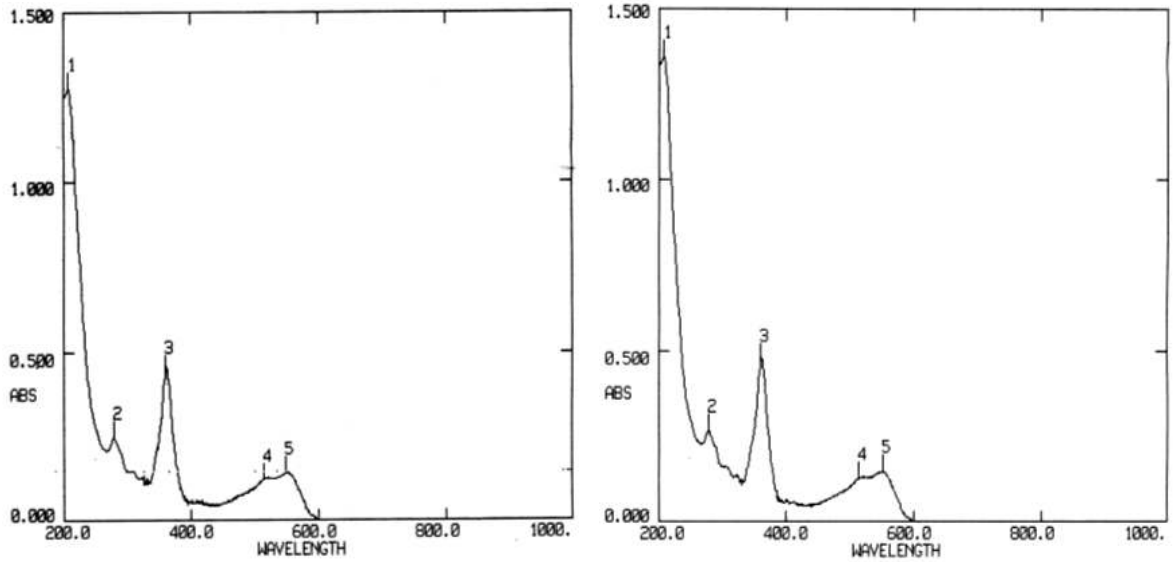
Different Sodium Chloride Concentrations: Different concentrations of sodium chloride had decreased the biosynthesis of vitamin B_{12} by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 5).

Different Yeast Extract Concentrations: The optimum concentration of yeast extract had a maximum biosynthesis of vitamin B_{12} could be recorded at 0.25 g/l by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 6).

Different $MgSO_4.7H_2O$ Concentrations: The optimum concentration of $MgSO_4.7H_2O$ for maximum biosynthesis of vitamin B_{12} could be recorded at 0.5 g/l by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig.7) .

Different K_2HPO_4 Concentrations: The optimum K_2HPO_4 concentrations for maximum biosynthesis of vitamin B_{12} could be recorded at 2.0 g/L by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 8) .

Different KCl Concentrations: The optimum concentration of KCl for maximum biosynthesis of



A) Vitamin B12 Produced by mixed cultures *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A, AZ- 8A.
B) Standard of vitamin B_{12}

Fig. 14: Ultra-violet spectrum of both produced vitamin B_{12} and Standard Vitamin B_{12}

vitamin B_{12} could be recorded at 1.0 g/l by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 9).

Different $CaCO_3$ Concentrations: The optimum concentration of $CaCO_3$ for maximum biosynthesis of vitamin B_{12} could be recorded at 4.0 g/l by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 10).

Different Carbon Sources: The galactose is the best carbon source for biosynthesis of vitamin B_{12} followed by mannose, glucose, xylose, starch, sucrose, arabinose, maltose and fructose respectively by using mixed cultures from *Streptomyces halstedii* and *Bacillus firmus* (fig. 11) .

Different Nitrogen Sources: The L-asparagin is the best nitrogen for the biosynthesis of vitamin B_{12} followed by sodium nitrate, ammonium chloride, glycin, potassium nitrate, ammonium sulphate, glutamic cid, argnine, beef extract and peptone in case of mixed culture of *Streptomyces halstedii* with *Bacillus firmus* (fig. 12).

Fermentation, Extraction, Crystallization and Purification of Vitamin B_{12}: Six discs from each microbial growth (*Bacillus firmus* and *Streptomyces halstedii*) were introduced aseptically into each sterile 250 ml Erlenmeyer flask containing 5.0 g dry weight of mixed agricultural wastes supplied with 30 ml mineral salt solution at optimum conditions for maximum vitamin production. The pH was adjusted at 10 and incubated at 35 ^{0}C for 96 hrs. The fermented medium was adjusted at pH 5.5. Potassium cyanide was added gradually to make a final concentration of 0.001% (w/w), and then extraction process was carried out. n-Butanol was added to fermentation broth at a ratio of 1:1 (v/v). The organic phase was collected, evaporated under reduced pressure using a rotary evaporator. The obtained residual extract of vitamin B_{12} was dissolved in 20 ml distilled water, and then treated with 100 g of activated carbon. After washing the activated carbon with water (120 ml), the adsorbed matter on the activated carbon was eluted with 75% aqueous acetone solution (100 ml). The eluted matter was condensed under reduced pressure to 2 ml.

Purification of the vitamin B_{12} has been carried out by silica gel column chromatography technique. Active fractions of vitamin B_{12} were concentrated and attained its maximum productivity at fraction No.19. Finally the purified compound was re-crystallized from the aqueous solution with acetone by cooling at zero °C for 12 hours (pink color compound was obtained) (fig. 13).

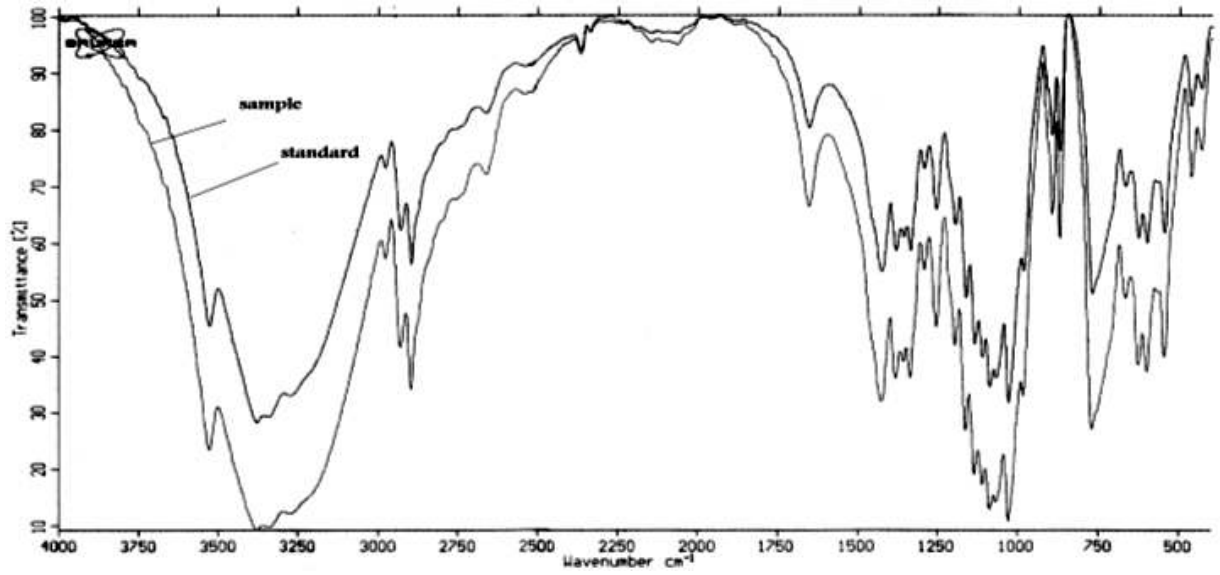
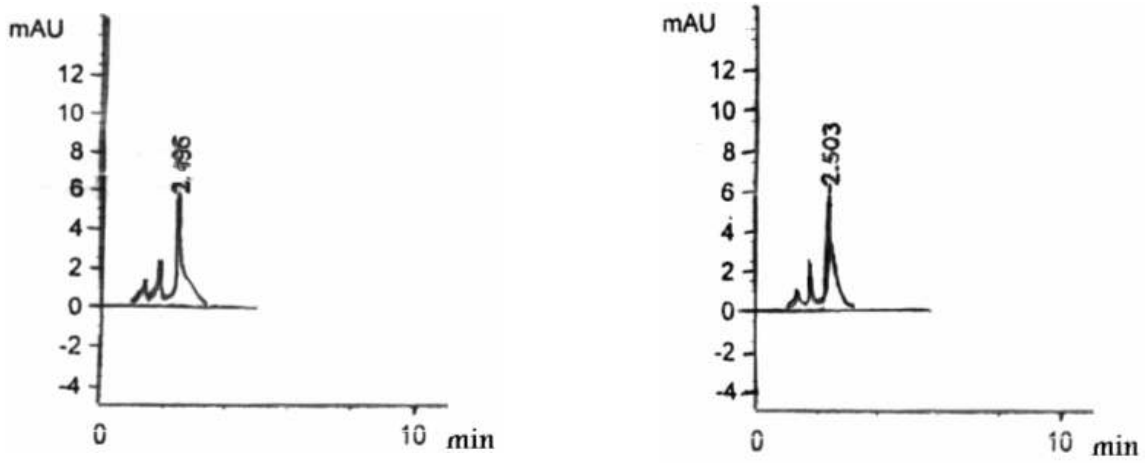


Fig. 15: Infra-Red spectrum of both produced vitamin B_{12} and Standard vitamin B_{12}



(A) **(B)**

A) Vitamin B_{12} Produced by *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A
B) Standard of vitamin B_{12}

Fig. 16: HPLC spectrum of both produced vitamin B_{12} and Standard vitamin B_{12}

Spectroscopic Characteristics: The spectroscopic analysis (UV, IR and HPLC spectrum) were used for comparative studies between vitamin B_{12} produced by mixed culture, *Bacillus firmus* and *Streptomyces halstedii* and authentic vitamin B_{12}.

Ultraviolet (UV) absorption spectrum of the compound recorded its maximum absorption peak at 360.0 nm while standard vitamin B_{12} at 361 (fig, 14). Infra red (IR) spectrum of the produced compound and standard vitamin B_{12} showed characteristic band corresponding to 19 peaks (fig, 15). HPLC- spectrum of compound showed its maximum peak at 2.503 while standard vitamin B_{12} was at 2.496 (fig, 16).

Discussion: Only two mixed cultures from *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A was found to produce significantly higher yield of the vitamin B_{12}.were screened for vitamin B_{12} biosynthesis by growing on medium used for microbial growth under solid state fermentation conditions (S.S.F) contained 3 gm of agricultural wastes supplemented with the mineral salts. Determination of vitamin B_{12}

productivity was carried out using *E.coli*, ATCC 14169 was giving 37.7 µg/ml. Similar result was obtained by[24] the productivity of vitamin B_{12} was giving 19.7 µg/ml).

For optimizing the biosynthesis of vitamin B_{12} from mixed cultures from *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ- 8A, different cultural conditions such as pH, temperature, incubation period and size inoculum. Furthermore study the effect of substrate concentrations, sodium chloride conc., yeast extratct conc., K_2HPO_4, $MgSO_4.7H_2O$, KCl, $CaCO_3$, different carbon and nitrogen sources was studied.

The maximum biosynthesis was achieved at the end of an incubation period of 96 hrs. for vitamin B_{12} biosynthesis using six discs of mixed cultures. Similar result had been recorded by[1].

The fact that maximum yield of the vitamin B_{12} occurred at the end of an incubation temperature of 35ºC at pH 10 were in complete accordance with those reported by[8,30,34,37].

Data of the effect of different carbon and nitrogen sources on the biosynthesis of vitamin B_{12} require galactose, asparagine, and required yeast extratct, K_2HPO_4, $MgSO_4.7H_2O$, KCl and $CaCO_3$ at concentrations 0.25 g/l, 2..0 g/l; 0. 5 g/l; 1.0 g/l; and 4.0 g/l respectively. Similar results have been recorded by various workers:[36,8,29,26,4,30,34,19]. The fermentation process was carried out for 96 hrs at 35 ^{0}C. The fermented broth was adjusted at pH 5.5. Potassium cyanide was added gradually to the metabolic product to make a final concentration of 0.001% (w/w) that convert of cobalamin to cyanocobalamin then extracted by n-butanol at pH 5.5. Similar results were obtained by[24,22].

Organic phase was collected and evaporated under reduced pressure using a rotary evaporator. Extract was concentrated and crystallized by dissolving in 20 ml distilled water that was then treated with 100 g of activated carbon. After washing the activated carbon with water (120 ml), the adsorbed matter on the activated carbon was eluted with 75% aqueous acetone solution (100 ml) pink-colored crystals were obtained. Similar results were recorded by[28,38,32,35].

The purification process through a column chromatography packed with silica gel and an eluting solvents composed of methanol: water: acetic acid (50:50:1 v/v), indicated that the maximum activity was occurred at fraction No. 19. Many workers were used a column chromatography technique for purification of vitamin B_{12}[27,40,32,25]. The spectroscopic analysis (UV, IR and HPLC spectrum) were used to perform comparative studies between vitamin B_{12} produced by two mixed cultures *Bacillus firmus* AZ-78B and *Streptomyces halstedii*, AZ-8A, and standard vitamin B_{12}. Ultraviolet (UV) absorption spectrum of the produced compound recorded its maximum absorption peak at 361 nm, while standard vitamin B_{12} was at 361 nm. Infra red (IR) spectrum of both the produced compound and standard vitamin B_{12} showed characteristic band corresponding to 19 peaks. HPLC- spectrum of the produced compound showed that the maximum peak was at 2.496, while for standard vitamin B_{12} was at 2.503). Similar result of ultraviolet (UV) absorption spectrum was conducted by[24,33,11,17,12,20,32,13,22].

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