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Production of vitamin B_{12} and folic acid from agricultural wastes using new bacterial isolates

Mostafa M. El-Sheekh[1]*, Ibrahim S. Abd-Elsalam[2], Samya Shabana[1] and Ahmed Zaki[1]

[1]Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt.
[2]Natural and Microbial Products Chemistry, National Research Center, Dokki, Cairo, Egypt.

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The rate and optimum conditions for production of vitamin B_{12} and folic acid from a mixture of agricultural wastes, rice bran and wheat straw was studied with new bacterial isolates, using solid-state fermentation (SSF). The isolates *Klebsiella pneumoniae* and *Citrobacter freundii* showed the highest yields of 38.74 and 45.21 µg/l for vitamin B_{12} and folic acid, respectively, from 200 g/l of the studied wastes. The optimum fermentation temperature for this production was 30°C for 3 days. This method is important to exploit the agricultural wastes which cause severe problems for the environment.

Key words: Bacteria, folic acid, solid-state fermentation, vitamin B_{12}.

INTRODUCTION

Solid state fermentation (SSF) is a processes involving microorganisms grown on solid or semi-solid substrates or supports. Monitoring this process requires the measurement of environmental parameters (temperature, pH, water content and activity) and the carbon cycle (biomass, substrate concentration, CO_2). However, given the complexity and heterogeneity of the solid medium, these variables are not easily accessible and measurable (Bellon-Maurel et al., 2003). Both vitamin B_{12} and folic acids are among the most essential vitamins for metabolism. They are involve in the formation of red blood cells and the activity of the central nervous system (Smith et al., 2007). In human, two enzymatic reactions are known to be dependent on vitamin B_{12} as a cofactor. In the first reaction, methylmalonic acid is converted to succinyl-CoA, while in the second reaction homocysteine is converted to methionine by using both vitamin B_{12} and folic acid as cofactors (Verhoef et al., 1996). On the other hand, folic acid is necessary for the synthesis of DNA (Kamen, 1997; Atta et al., 2008).

Different trials have been made for the production of vitamin B_{12} (Okada et al., 1985; Wang et al., 2011). The production of B_{12} and folic acid have been obtained at relatively low concentrations (1.8-41.4 ng/g) using soybean as solid substrate. Atta et al. (2008) showed that the productivity of B_{12} was enhanced using a mixture of substrates. Herranen et al. (2010) have isolated 42 bacterial strains from oat bran and rye flask, only 26 of these strains have the ability to produce folic acid, and belong to the genera *Bacillus*, *Actinobacter* and *Staphylococcus*.

This study investigated the application of new bacterial isolates in the production of vitamins B_{12} and folic acid and finding the suitable agricultural wastes, as substrates, using the solid-state fermentation (SSF).

MATERIALS AND METHODS

Isolation, screening and biochemical characterization of the tested bacteria

Different fecal bacteria have been isolated from different stool samples and sewage water and biochemically identified according to Bergey's Manual (1986). The screening and the biochemical

*Corresponding author. E-mail: mostafaelsheekh@yahoo.com.

characterization of the tested bacteria for the production of vitamin B_{12} and folic acid were estimated. In addition to the isolated bacteria, the fungus *Rhizopus stolonifer* NRC 223 was kindly provided by Natural and Microbial Products Department, National Research Center (NRC), Cairo, Egypt.

Reagents

Kovac's reagent

Paradimethyl aminobenzaldehyde (5.0 g) was dissolved in 25 ml of conc. HCl (35%) then 75 ml amyl alcohol were added and agitated for 10 min. The reagent was stored in closed vials at 4°C.

Methyl red

Methyl red dye (0.1 g) was dissolved in 300 ml of ethanol and raised up to 500 ml with distilled water.

Voges-Proskauer test (V-P) reagent

This is a solution of 5% alcoholic α-naphthol (5 ml α -naphthol in 95% alcohol) and 40% potassium hydroxide. The tubes were incubated at 35°C (water bath) for 4 h. Two drops of Creatine reagent were added with gentle mix, then 3 drops of α-Naphthol reagent were added with gentle mix. Three drops of 40% Potassium Hydroxide were added and gently mixed for 10 s. The tubes were allowed to stand for 15 min before interpreting the color.

Reagents for radio-immuno assay kit

All reagents needed for determination of vitamin B_{12} and folate were included in a commercial kit derived from MP Biomedicals, Diagnostics Division, New York, USA.

Ditiothreitol solution (DTT)

This solution contains ditiothreitol in phosphate buffer with stabilizer (Herbert et al., 1984).

Vitamin B_{12}/Folate Tracer: Contains < 1.5 µCi (55.5 kBq) [^{57}Co] vitamin B_{12} and < 3 µCi (111 kBq) [^{125}I] Folate in borate buffer with human serum albumin, dextran, potassium cyanide, endogenous binder blocker, dye and preservative. Cultivation process is as described by Herbert et al. (1984).

Inoculum preparation

Pure bacterial and fungal slants were suspended in 100 ml sterile distilled water. The suspension was used to inoculate Erlenmeyer flasks (250 ml) containing the selected agricultural waste.

Substrate preparation

Different plant wastes were used as fermentation substrates for vitamin B_{12} production process. These wastes included rice bran, rice straw, wheat bran, wheat straw, clover straw, corn stem, soy bean and cane mash. These highly lignified substrates were pretreated by cutting, grinding and alkaline hydrolysis, which separated the lignin components of the waste from ligno-cellulose and nitrogenous sources (Abd El-Hamid et al., 1997; Wanapat et al., 2009). Also, Beat molasses were tested as substrates through dilution with tap water to approximately 20% sugar concentration, centrifugation at 2500 rpm to remove the suspended matter and finally supplemented with 0.05 g% $(NH_4)_2SO_4$, before autoclaving at 121°C for 20 min.

Solid-state fermentation

Cultivation of the isolates was carried out in 250 ml Erlenmeyer flasks, each containing 20 g of the solid substrates and 100 ml of distilled water. Then, autoclaved and inoculated by $1x10^6$ spore/ml of the fungus and $4.5x10^8$ cell/ml of the selected bacteria (Quesada-Chanto et al., 1994; Atta et al., 2008). Different weights (20, 40, 75, 150, 200 and 250 g/l) were used separately as substrates for vitamin productions. The flasks were incubated statically at different temperatures for different selected fermentation periods. The contents of each flask were centrifuged at 4000 rpm for 10 min to separate the growing isolates from the culture media. The yields of both vitamin B_{12} and folate were determined relative to the amount of consumed substrate dry weight.

Determination of vitamin B_{12} and folic acid

Radio-immuno assay (RIA) was used for the simultaneous quantitative determination of vitamin B_{12} and folic acid according to the method of Herbert (1999). In this procedure, endogenous sample binders for both vitamin B_{12} and folic acid were destroyed after incubation with tracer/dithiothreitol for 15 min followed by a 10 min extraction at alkaline conditions pH (12-13). This method avoided the need for heating the sample at 100°C.

Statistical analysis

All the experiments were done in a triplicate ± standard deviation (SD). Statistical analyses were applied with the software SPSS version 10.0 for windows (SPSS Inc., 1999). The obtained data were statistically analyzed using analysis of variance (ANOVA) to determine the degree of significance for the variations tested, the least significant differences test (LSD) and the F test at $p = 0.05$ as significant level (Bishop, 1983).

RESULTS AND DISCUSSION

Screening for different bacterial isolates

Some fecal bacteria strains have been used for the production of vitamin B_{12} and folic acid using rice bran as a substrate. The fermentation process continued for 3 days at 30°C and an initial pH of 8. The culture filtrate was assayed for vitamin B_{12} and folic acid. The final pH value and substrate consumption were also determined. Nine bacterial isolates were screened for their ability to produce vitamin B_{12} and folic acid (Table 1). It was found that *Klebsiella pneumoniae* and *Citrobacter freundii* produced a considerable yield of the target vitamins. Accordingly, *K. pneumoniae* and *C. freundii* were selected for further investigations. Our results supported the concept that fecal bacterial flora are one of the major producers of vitamin B_{12} and other vitamin B complex (Hill, 1997). Herranen et al. (2010) was able to isolate 42

Table 1. Bacterial isolates and the concentration of vitamin B_{12} and folate (µg/l), substrate consumption (%) and final pH.

Bacterial isolate	Vitamin B_{12}	Folate concentration	Substrate consumption	Final pH
Pseudomonas aeruginosa	4.12±0.5	20.37±0.8	5±1.0	4.9±0.3
Klebsiella pneumoniae	6.77±2.2	25.31±0.9	6±1.5	5.0±0.1
Escherichia coli	5.40±0.6	13.37±1.6	4±1.0	4.5±0
Shigella sp.	1.68±0.3	9.87±0.4	2±0.0	4.6±0.2
Salmonella enteritidis	0.50±0.1	8.20±0.5	2±0.5	4.3±0.2
Protieus vulgaris	3.85±0.3	20.35±0.7	5±1.0	5.7±0.1
Serratia marcescens	1.07±0.7	14.91±3.1	4±1.0	4.6±0.2
Citrobacter freundii	5.67±0.2	21.03±0.7	5±1.0	5.1±0.1
Enterobacter cloacae	3.66±0.7	14.32±0.6	4±1.5	5.3±0.1

Initial pH: 8.0, incubation for 3 days at 30°C, Substrate type: Rice bran, Substrate weight: 75 g/l.

Table 2. Biochemical characteristics of some bacterial isolates.

Biochemical character	*Sa.*	*Es.*	*Ps.*	*Sh.*	*Se.*	*Kl.*	*Pr.*	*Ci.*	*En.*
Gram stain	-	-	-	-	-	-	-	-	-
Motility	+	+	+	-	+	-	+	+	+
Growth on Mac-Conkey agar	+	+	+	+	+	+	+	+	+
Growth on EMB with metallic sheen	-	+	-	-	-	-	-	-	-
Growth on SS medium	+	-	-	+	-	-	-	-	-
Fluorescent pigment production on King'B medium	-	-	+	-	-	-	-	-	-
Indole production	-	+	+	-/+	-	-	+	-	-
Methyl red test	+	+	+	-	-	+	+	+	-
Voges-Proskauer test	-	-	+	+	+	+	-	-	+
Citrate utilization	+	-	+	-	+	+	-	+	+
H_2S production	+	-	-	+	-	-	+	-	-
Urease production	-	-	-	-	-	+	+	+	+
Gas production from glucose	+	+	-	+	+	+	+	+	+
Acid production from glucose	+	+	-/+	-	-	+	+	+	+
Lactose fermentation	-	+	+	+	-	+	-	+	+
DNase production	-	-	-	-	+	-	-/+	-	-
Oxidase production	-	-	+	-	-	-	-	-	-
O-F test	+	+	+	-	+	-	-	+	-

+ indicate positive results; - indicate negative results; -/+ indicate 50% results. *Sa.*= *Salmonella* sp.; *Es.*= *Escherichia coli; Ps.*= *Pseudomonas* sp.; *Sh.*= *Shigella* sp.; *Se.*= *Serratia* sp.; *Kl.*= *Klebsiella* sp.; *Pr.*=*Protieus* sp.; *Ci.*= *Citrobacter* sp.; *En.*= *Enterobacter* sp.

bacterial strains from soya and showed that 26 of them had the ability to produce folic acid.

Biochemical characteristics of some bacterial isolates

The identification of the tested bacteria has been carried out. The results presented in Table 2 revealed to some important biochemical characters of some bacterial isolates and their identification**.**

Effect of different agricultural waste substrates on the production of vitamin B_{12} and folic acid

The selected two bacterial isolates, *Klebsiella pneumoniae* and *Citrobacter freundii,* were allowed to grow on different agricultural wastes as substrates. The fermentation process was continued for 3 days at 30°C. The culture filtrate was assayed for vitamin B_{12} and folic acid contents.

From Figures 1 and 2, it was noted that the rice bran and wheat straw gave the highest yields of both vitamin B_{12} and folic acid. *K. pneumoniae* could produce 6.77 and 25.31 µg/l of vitamin B_{12} and folic acid, respectively, from 6% consumed rice bran compared with the amounts of 3.44 and 18.75 µg/l for the vitamin B_{12} and folic acid, respectively, produced from 5% consumed wheat straw. On the other hand, *C. freundii* produced 5.67 µg/l of vitamin B_{12} and 21.03 µg/l of folic acid from rice bran, while producing 4.17 µg/l of vitamin B_{12} and 14.96 µg/l of

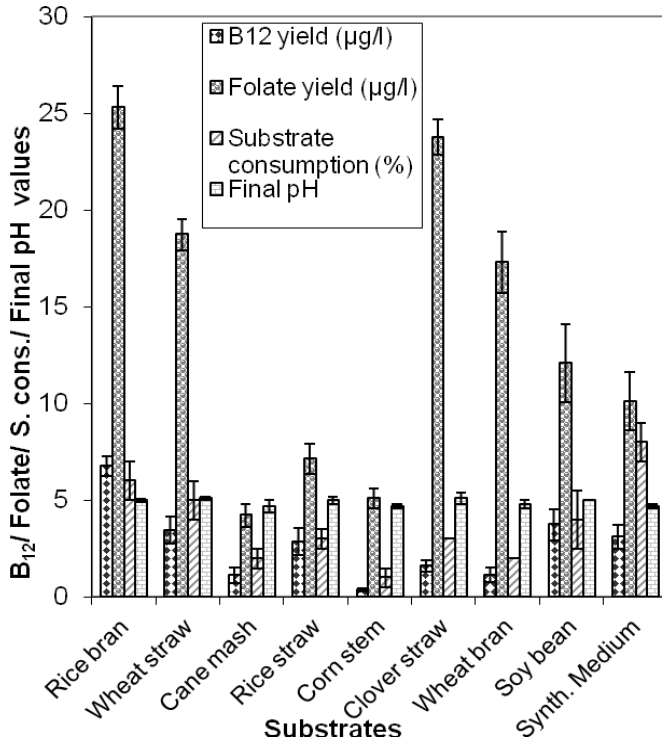

Figure 1. Effect of different substrate types on the production of vitamin B_{12} and folic acid by *Klebsiella pneumoniae*.

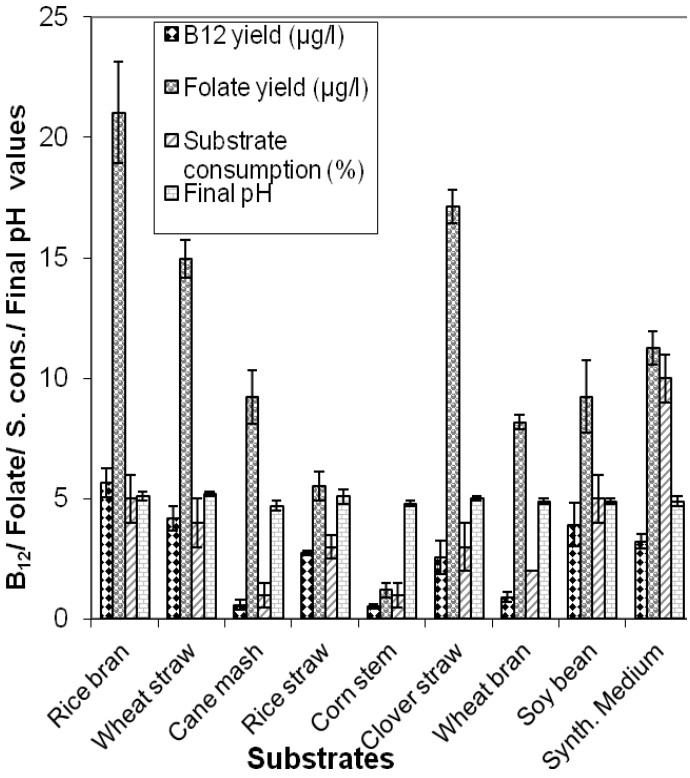

Figure 2. Effect of different substrate types on the production of vitamin B_{12} and folic acid by *Citrobacter freundii.*

Table 3. Effect of different substrate weights on the production of vitamin B_{12} and folic acid by *Klebsiella pneumonia.*

Substrate weight (g/l)	Vitamin B_{12} conc. (µg/l)	Folic acid conc. (µg/l)	Substrate consumption (%)
20	3.23±0.2	10.34±0.1	5±1
40	5.13±0.3	14.28±0.5	6±1.0
75	14.22±0.9	30.77±1.5	8±0.5
150	18.22±0.9	37.87±0.2	9±0.0
200	19.67±0.6	39.13±1.1	9±1.0
P value	***	***	**
F test	302	628	16
LSD (at 0.05)	1.25	1.49	1.41

Initial pH: 8, incubation for 3 days at 30°C, substrate: Wheat straw + Rice bran (ratio 1:2). *** = Highly significant ($P \leq 0.001$); ** = Moderately significant ($P < 0.01$); NS = Non-significant ($P > 0.05$).

Table 4. Effect of different substrate weights on the production of vitamin B_{12} and folic acid by *Citrobacter freundii.*

Substrate weight (g/l)	Vitamin B_{12} conc. (µg/l)	Folic acid conc. (µg/l)	Substrate consumption (%)
20	2.44±0.7	8.21±0.3	6±0.4
40	3.58±0.9	11.87±0.2	5±0.5
75	10.93±0.5	17.65±0.3	6±1
150	14.07±0.1	30.81±0.2	8±1
200	14.3±0.2	33.13±1	9±1
P value	***	***	**
F test	270	1079	12
LSD (at 0.05)	0.99	1.01	1.70

Initial pH: 8, incubation for 3 days at 30°C, substrate: Wheat straw + Rice bran (ratio 1:2).; *** = Highly significant ($P \leq 0.001$); ** = Moderately significant ($P < 0.01$); * = Significant (P = 0.01 – 0.0).

folic acid from wheat straw. These results are in agreement with that stated by Okada et al. (1985).

Suitability of different substrates weights on the production of vitamin B_{12} and folic acid

The effect of different agricultural substrate weights (20, 40, 75, 150 and 200, 250 g/l) on the production of vitamins B_{12} and folic acid were investigated. The data in Tables 3 and 4 showed that the best B_{12} and folic acid out puts (19.67 and 39.13 µg/l) respectively, were obtained at 200 g/l by using *Klebsiella pneumoniae*. Consequently, *Citrobacter freundii* produced the maximum yields of B_{12} and folic acid, 14.3 and 33.13 µg/l, respectively at the same substrate weight.

Effect of different incubation periods on the production of vitamin B_{12} and folic acid

The selected two bacterial isolates were allowed to grow on the fermentation medium at different incubation periods (1, 2, 3, 4 and 5 days). The results presented in Figures 3 and 4 showed that *Klebsiella pneumoniae* produced the maximum amount of vitamin B_{12} and folic acid, 38.74 and 45.21 µg/l, respectively at the third day of fermentation. However, *Citrobacter freundii* showed decrease in vitamin B_{12} production (19.37 µg/l) as compared to *Klebsiella pneumoniae* on the third day, while the yield of folic acid was 36.32 µg/l with 10% substrate consumption at the same day. On the other hand, at the longer fermentation periods (4 and 5 days), the productivity of both vitamins were considerably reduced by the two isolates. Atta et al. (2008) found that the maximum biosynthesis of B_{12} was obtained on the 4th day of incubation period. The current incubation period for maximum production of the target vitamins was in agreement with that recorded by Okada et al. (1985). The results of the statistical analysis revealed that the fermentation time was highly significant ($P<0.001$) for vitamins B_{12} and folic acid yields and substrate consumption for both selected bacterial isolates.

Effect of different fermentation temperature on the production of vitamin B_{12} and folic acid

The production of the desired products were carried out at different fermentation temperatures (20, 25, 30, 35 and

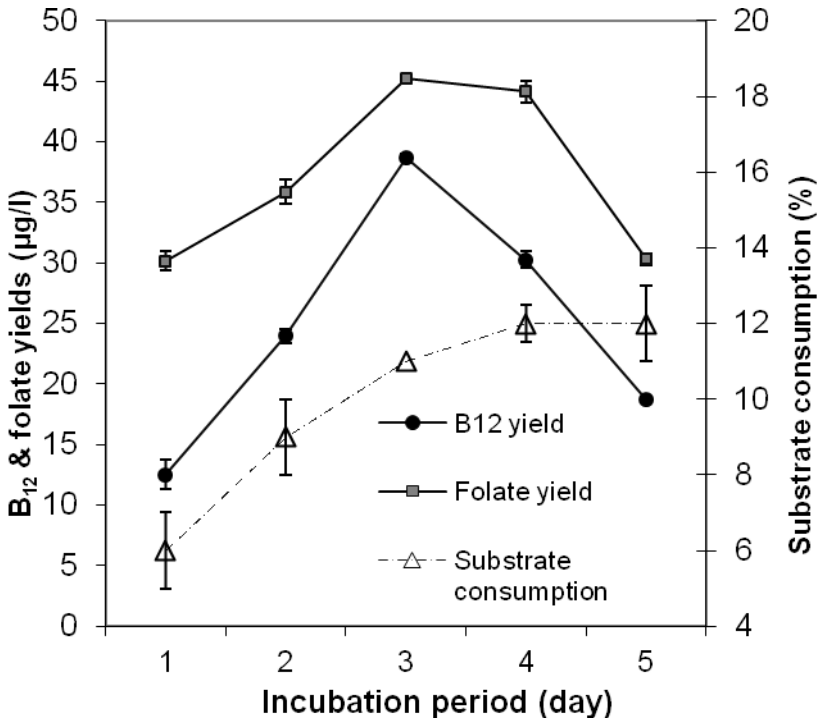

Figure 3. Effect of different incubation periods on the production of vitamin B_{12} and folic acid by *Klebsiella pneumoniae*. Initial pH: 8.0, Temperature 30°C, Substrate type: Rice bran, Substrate weight: 75 g/l.

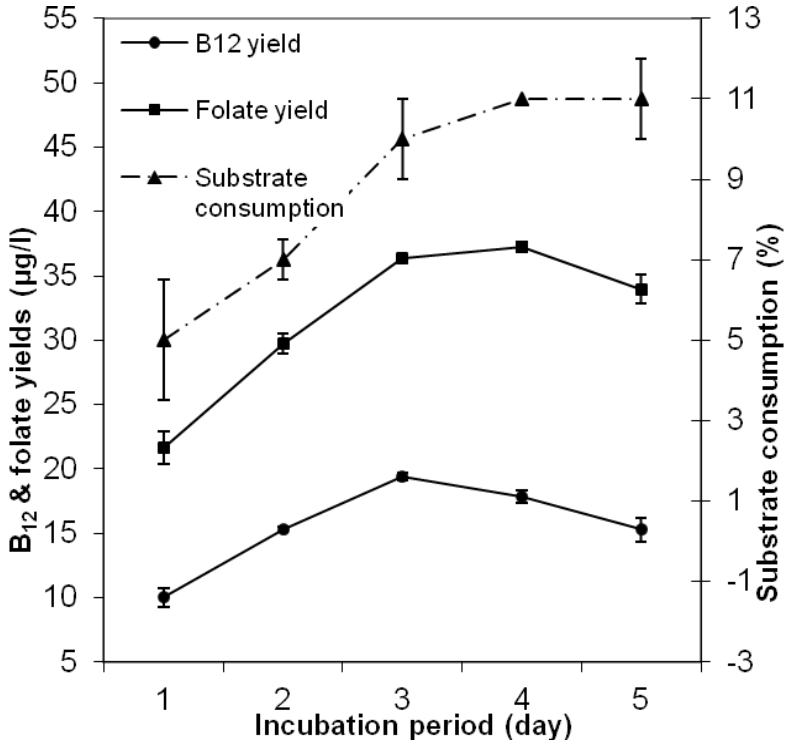

Figure 4. Effect of different incubation periods on the production of vitamin B12 and folic acid by *Citrobacter freundii*. Initial pH: 8.0, Temperature 30°C, Substrate type: Rice bran, Substrate weight: 75 g/l.

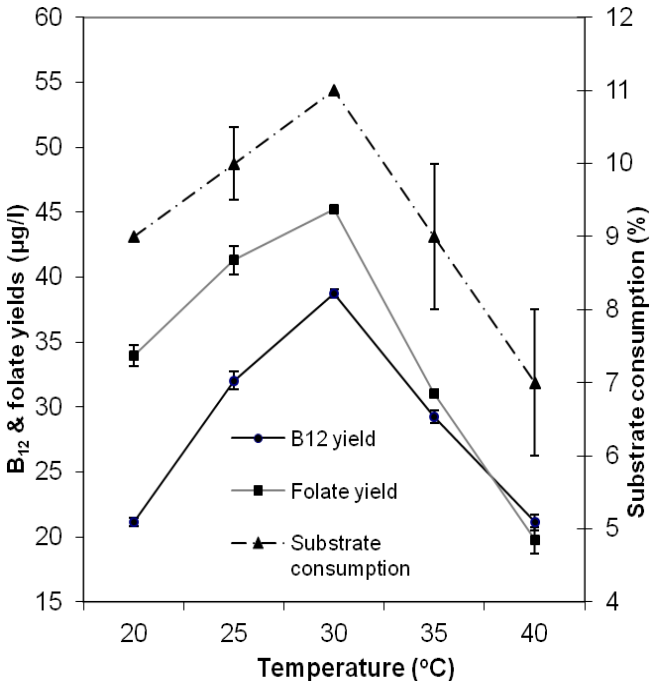

Figure 5. Effect of different temperatures on the production of vitamin B_{12} and folic acid by *Klebsiella pneumoniae.* Initial pH: 8.0, incubation for 3 days, Substrate type: Rice bran, Substrate weight: 75 g/l.

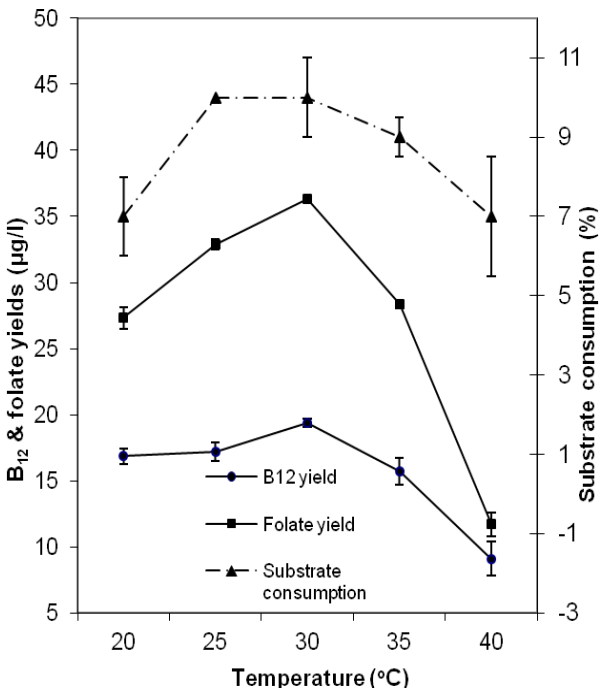

Figure 6. Effect of different temperatures on the production of vitamin B_{12} and folic acid by *Citrobacter freundii.* Initial pH: 8.0, incubation for 3 days, Substrate type: Rice bran, Substrate weight: 75 g/l.

40°C) using the above selected microorganisms. The results presented in Figures 5 and 6 revealed that the maximum B_{12} and folic acid out puts (38.7 and 45.21 µg/l, respectively) were obtained at 30°C using *Klebsiella pneumoniae*. However, the productivity of B_{12} and folic acid (19.3 and 36.32 µg/l, respectively), were also obtained at the same fermentation temperature using *Citrobacter freundii*. Our results are in agreement with the data obtained by Atta et al. (2008) who found that the maximum B_{12} could be achieved at 35°C. The results of the statistical analysis revealed that the fermentation temperature was highly significant (*P< 0.001*) for vitamins B_{12} and folic acid yields and substrate consumption for both selected bacteria.

CONCLUSION

The ability of some bacterial isolates specially the selected isolates showed great ability to consume the agricultural wastes as substrates to produce vitamins, consequently; it helps in using low cost source for production and decreasing the environment pollution. The incubation time and temperature were found to be highly significant in the production process.

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