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Mathematical modeling of some medium constituents and its impact on the production of vitamin B_{12} and folic acid by *Klebsiella pneumoniae* under solid state fermentation

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 Department, National Research Center (NRC), Dokki, Cairo, Egypt.

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The present investigation involved testing the effect of different carbon sources (glucose, sucrose and starch) as well as different nitrogen sources (peptone, yeast extract and urea) on the production of vitamin B_{12} using *Klebsiella pneumonia* grown on mixture of agriculture wastes (wheat straw and rice bran). The results show that glucose was the best carbon source compared to the other di- and polysaccharides used. The best vitamin B_{12} output (63.87 µg/L) was obtained at 3 g/L glucose. On the other hand, peptone appeared to be the most suitable nitrogen, since it gave (57.8 µg/L) of B_{12} compared to the other nitrogen sources used. Statistical analysis for the obtained results had been carried out and the data showed that glucose and peptone additions were highly significant compared to the other sources under investigation.

Key words: Vitamin B_{12} production, solid state fermentation (SDF), bacteria.

INTRODUCTION

Vitamin B_{12} like other B vitamins is important for the metabolism. It helps in the formation of red blood cells and in the maintenance of the central nervous system (Wickramasinghe, 1995). The deficiency of vitamin B_{12} is associated with hematologic, neurologic and psychiatric manifestations. It is also a common cause of megaloblastic anemia (Atta et al., 2008). The deficiency may exert indirect cardiovascular effects in addition to the hematologic and neuropychatric manifestations (Nygard et al., 1997; Kałużna-Czaplińska et al., 2011).

On the other hand, folic acid is necessary for fertility in both men and women. In men it contributes to spermatogenesis but in women enhance oocycte maturation placentation (Ebisch et al., 2007). The biosynthesis of this essential nutrient is intricate, involved and, remarkably, confined to certain members of the prokaryotic world, seemingly never have to have made the eukaryotic transition (Martens et al., 2002). Bacteria are known for its de novo synthesis of vitamin B_{12} and folic acid through biological and biotechnological pathways. Masuda et al.

*Corresponding author. E-mail: mostafaelsheikh@science.tanta.edu.eg.

Table 1. Effect of different glucose concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Glucose concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	45.37 ± 0.7	50.91 ± 1.1	11 ± 1.0
2.00	56.03 ± 0.3	54.43 ± 0.8	12 ± 0.5
3.00	63.87 ± 0.9	58.96 ± 0.5	13 ± 1.0
4.00	60.28 ± 0.6	63.88 ± 0.2	13 ± 0.5
5.00	55.84 ± 2.3	61.72 ± 1.0	12 ± 0.0
P value	***	***	**
F test	226	281	5.8
LSD at 0.05	1.94	1.30	1.15

Initial pH 8. Incubation time 3 days, temp. 30ºC, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L.*** Highly significant $p<0.001$,** Moderate significant *p*<0.*01*, Control without addition.

(2012) investigated the extracellular production of folate, vitamin B12, and thiamine in cultures of lactic acid bacteria (LAB) isolated from nukazuke, a traditional Japanese pickle. They studied the relationships between the vitamin production and properties of LAB as tolerance to salts, ethanol, etc. Among 180 isolates of LAB, two strains were capable to produce 100 µg/L vitamins.

For the above mentioned factors, this work aims at the production of B_{12} and folic acid using some raw materials involving different agriculture wastes for the magnification of the production process.

MATERIALS AND METHODS

Microorganisms

The microorganism used in the current work *Klebsiella pneumoniae* was isolated from fecal specimen and identified in a previously discussed work (El-Sheekh et al., 2013). The fungus *Rhizopus nigricans* was provided from Natural and Microbial Products Department, National Research Center, (NRC) Dokki, Cairo.

Substrate preparation

The agriculture wastes (wheat straw and rice bran) were used in a mixture of 1:2 (w/w) respectively. These wastes were pretreated by cutting, grinding and alkaline hydrolysis with sodium hydroxide to separate the lignin components (Wanapate et al., 2009; El-Sheekh et al., 2013).

Cultivation

Erlenmeyer flasks (250 ml) each contain 20 g of the solid substrate and 100 ml sterile distilled water, which were previously inoculated with 1×10^6 spores of *Rhizopus*/ml and 4.5×10^8 bacterial cell/ml, were incubated statistically at 30°C for three days and the initial pH was adjusted at 8 (Keuth and Bisping, 1994; Atta et al., 2008; El-Sheekh et al., 2013). The flasks contents filtered and the filtrate was centrifuged at 4000 rpm for 10 min to separate the substrate from the culture medium. The substrate consumed was also determined after each experiment by calculating the percentage of the difference of weight before and after the experiment.

Estimation of vitamin B_{12} and folic acid

Radio-immunoassay (RIA) was used for the simultaneous quantitative determination of vitamin B_{12} and folic acid in comparison with standard according to the method described by Akatsuka and Atsuya (1989) and Wongyai (2000).

Statistical analysis

The obtained results were statistically analyzed using the analysis of variance (ANOVA) to determine the degree of significance for the variation of both vitamin B_{12} and folic acid yields. F test and the least significance different (LSD) at 0.05 level was also calculated for the mean values.All the statistical methods were applied according to the method described by Bishop (1983).

RESULTS AND DISCUSSION

Suitability of different carbon sources

Glucose concentration

Different glucose concentrations (1, 2, 3, 4 and 5 g/L) were tested in the production process of vitamin B_{12} by *Klebsiella pneumoniae*. It was clearly noticed in Table 1, that the addition of glucose enhanced the production of vitamin B_{12} and folic acid. The highest yield of vitamin B_{12} (63.87 µg/L) was obtained at 3 g/L. Further increase in glucose concentration was accompanied by reduction in the yield. The results of the statistical analysis of glucose addition were highly significant at $p<0.001$ for vitamin B_{12} and folic acid.

Sucrose concentration

The addition of different sucrose concentrations (1, 2, 3,

Table 2. Effect of different sucrose concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Sucrose concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	38.74 ± 0.2	46.72 ± 0.8	11 ± 0.5
2.00	43.03 ± 1.0	49.39 ± 0.4	11 ± 1.0
3.00	47.12 ± 0.4	50.90 ± 0.5	11 ± 0.5
4.00	48.27± 0.3	51.77 ± o.4	12 ± 1.5
5.00	46.93 ± 0.9	51.08 ± 0.2	12 ± 1.0
P value	***	***	NS
F test	102	98	1
LSD at 0.05	1.07	0.82	1.58

Initial pH 8. Incubation time 3 days, temp. 30 °C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant p<0.001,**Moderate significant p<0.01, Control without addition.

Table 3. Effect of different starch concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Starch concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	40.88 ± 0.9	50.27 ± 0.3	11 ± 1.0
2.00	42.27 ± 0.6	50.79 ± 0.2	11 ± 1.5
3.00	43.93 ± 0.4	51.61 ± 0.5	12 ± 1.0
4.00	43.47 ± 0.3	51.13 ± 0.2	12 ± 0.5
5.00	42.89 ± 0.8	49.07 ± 1.0	12 ± 0.0
P value	***	***	NS
F test	31	68	1.2
LSD at 0.05	1.06	0.88	1.5

Initial pH 8. Incubation time 3 days, temp. 30°C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. *** Highly significant *p<0.001*, NSnon significant (p>*0.05*), control without addition.

4 and 5 g/L) were tested for vitamin B_{12} and folic acid production by *Klebsiella pneumoniae*. The results presented in Table 2 showed that the best Vitamin B_{12} yield (48.27 µg/L) was obtained at sucrose concentration 4 g/L. On the other hand, the folic acid yield was 51.77 µg/L at the same concentration.Further increase in the sucrose concentration was accompanied by slightdecrease in the outputs .The statistical analysis of sucrose addition showed that it was highly significant (p<0.001) for vitamin B_{12} and folic acid (Keuth and Bisping, 1993; Herranen et al., 2010).

Starch addition

The addition of different starch concentrations (1, 2, 3, 4 and 5 g/L) were investigated. The data in Table 3 revealed that the addition of this polysaccharide has low effect on the stimulation of the vitamin B_{12} and folic acid production. The maximum yields of vitamin B_{12} and folic acid were obtained at 3 g/L (43.93 and 51.61 µg/L), respectively. The statistical analysis of the sucrose addition results showed that it is highly significant (p<0.001) for B_{12} and folic acid, but non-significant at p>0.05 for the substrate used (Keuth and Bisping, 1994; Nygard et al., 1997; El-Sheekh et al., 2013).

Suitability of different nitrogen sources

Addition of different nitrogen sources (yeast extract, peptone and urea) were tested for their effects on the production of vitamin B_{12} and folic acid.

Yeast extract

In this experiment different concentrations of yeast extract (1, 2, 3, 4 and 5 g/L) were used to test the optimum production of vitamin B_{12} and folic acid. The results presented in Table 4 showed that the addition of 4 g/L enhanced the production of vitamin B_{12} and folic acid (46.76 and 55.51 µg/L) respectively, and accompanied by 13% substrate consumption (Keuth and Bisping, 1994; Nygard et al., 1997). The statistical analysis results revealed that the addition of yeast extract appeared to be highly significant (p>0.001) for both vitamin B_{12} and folic

Table 4. Effect of different yeast extract concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Yeast extract concentration (g/L)	Vitamin B_{12} content (µg/L)	Folicacid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	38.96 ± 1.1	52.74 ± 0.8	11 ± 1.0
2.00	43.00 ± 0.6	54.59 ± 0.4	12 ± 0.5
3.00	45.95 ± 0.8	54.63 ± 0.3	12 ± 1.0
4.00	46.67 ± 0.2	55.51 ± 0.6	13 ± 1.0
5.00	45.13 ± 0.5	51.69 ± 1.6	12 ± 1.5
P value	***	***	NS
F test	85	67	1.9
LSD at 0.05	1.17	1.43	1.70

Initial pH 8. Incubation time 3 days, temp. 30 °C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant $p<0.001$, NS non significant ($p>0.05$), Control without addition.

Table 5. Effect of different peptone concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Peptone concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
Control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
1.00	43.23 ± 0.4	50.08 ± 1.8	12 ± 0.5
2.00	47.61 ± 0.9	57.11 ± 0.8	13 ± 0.0
3.00	55.09 ± 0.7	60.87 ± 0.3	14 ± 1.0
4.00	57.33 ± 0.5	62.63 ± 0.4	14 ± 1.0
5.00	58.72 ± 0.6	61.91 ± 0.5	13 ± 1.5
P value	***	***	**
F test	556	207	5.5
LSD at 0.05	1.07	1.53	1.54

Initial pH 8. Incubation time 3 days, temp. 30 ^{0}C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant $p<0.001$, **Moderate significant $p<0.01$, Control without addition.

acid, but non-significant ($p<0.05$) for the substrate.

Peptone

Different concentrations of peptone (1, 2, 3, 4, and 5 g/L) were added to the fermentation medium after being sterilized. The results in Table 5 showed that addition of peptone at concentration 5 g/L to the medium, the best yield of vitamin B_{12} was 58.72 µg/L but the maximum folic acid output was 62.63 µg/L at 4 g/L as well as 14%v substrate consumption. The results of the statistical analysis revealed that the addition of peptone was highly significant ($p<0.001$) for B_{12} and folic yields.

Urea

The investigation was extended to study the effect of different concentrations of urea (0, 5, 1, 2 and 3 g/L) on the production of B_{12} and folic acid. The results presented in Table 6 showed that the best vitamin B_{12} output (47.33 µg/L) was obtained at 0.5 g/L, while, the maximum folic acid output (77.32 µg/L) was obtained at 2 g/L, (Keuth and Bisping, 1993; Herranen et al., 2010). The data of statistical analysis of the addition of urea was highly significant ($p<0.001$) for B_{12} and folicacid.

Conclusion

This study revealed that the productivity of vitamin B_{12} and folic acid was affected by both the carbon and nitrogen sources. The results showed the priority of using glucose and yeast extract which induced highly significant effect on the production process.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Table 6. Effect of different urea concentrations on production of vitamin B_{12} and Folic acid by *Klebsiella pneumoniae.*

Urea concentration (g/L)	Vitamin B_{12} content (µg/L)	Folic acid content (µg/L)	Substrate consumption (%)
control	38.74 ± 0.3	45.21 ± 0.2	11 ± 0.0
0.5	47.33 ± 0.9	46.33 ± 0.2	12 ± 1.5
1	44.12 ± 0.5	59.87 ± 0.7	12 ± 0.5
2	31.23 ± 1.2	77.32 ± 0.8	13 ± 1.0
3	20.23 ± 1.4	69.21 ± 1.1	11 ± 1.0
P value	***	***	NS
F test	390	1199	2.3
LSD at 0.05	1.74	1.28	1.73

Initial pH8. Incubation time 3 days, temp. 30°C, substrate W.S+R.B (ratio 1:2 W/W) substrate weight 200 g/L. ***Highly significant p<0.001, NSnon significant (p>0.05), Control without addition.

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