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Production of Vitamin B_{12} and Folate Using a Potent Mutant Strain of *Klebsiella pneumonia*

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THE PRODUCTION of vitamin B_{12} and folate under solid state fermentation was investigated by using different mutants of *klebsiella penumonia*. The results showed that the isolate no (EM33TE) produce the maximum B_{12} and folate yields (77.45 and 88.22 ug/ml) respectively. The supplementation of the fermentation medium by different carbon sources (glucose, fructose, mannose, sucrose, maltose, lactose,starch and dextrin) indicated that the best carbon source was glucose and the maximum B_{12} yield (356.9ug/ml) was obtained by the sequential addition of glucose(10g) to the fermentation medium. PCR studies revealed that clear differences at bands number and size between the original strain and its mutants using RAPD analysis by three different random primers, these differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *K. pneumonia* genome after EMS-mutagenesis.

Keywords: Vitamin $B_{12,}$ Folate, EMS-mutagenesis, Solid state fermentation, RAPD analysis.

Introduction

Vitamin B_{12} is an important biological compound which appeared to be active as haemato-biotic factor in mammals and growth for many microbial and animals species. Vitamin B_{12} and folic acid are important in the formation and regeneration of red blood cells thus preventing anemia, also B_{12} is important as a dietary supplement for animals and human beings. Since the chemical synthesis of vitaminB_{12} requires more than 70 steps [1,2], the industrial production by chemical method and subsequent purification steps made it technically too difficult, expensive, unsafe to the operators and the process is not eco-friendly. Therefore, Vitamin B_{12} has been produced on an industrial scale using the batch or fed-batch process of microbial fermentation[3].

Various microorganisms including those of the genera *Streptomyces*, *Bacillus*, *Methanobacterium*, *Pseudomonas*, *Klebsiella* and *Propiono-bacterium* have been used to produce vitamin B_{12} on an industrial scale [4]. Among the abovestated microorganisms, *klebsiella* species are preferred for the production of vitamin B_{12}.

The present study gave insight about the enhancement of vitamin B_{12} and folate production. Random mutagenesis has been used in anticipation to generate mutant strains which can positively produce high yield of product. In general a high yield of vitamin B_{12} has been achieved by treating the microorganisms with mutagenic agents such as UV light or chemicals and selecting the strains with practical advantages, involving high productivity and genetic stability. Moreover, the genetic effect of EMS-mutagenesis on nucleotide sequence by random amplified polymorphic DNA (RAPD) analysis is also considered.

Materials and Methods

Materials

Microorganisms

Rhizopusstolonifer NRC and *K. pneumoniae* were obtained from Natural and Microbial Products Chemistry Department. The mold was added to the sterile waste before fermentation to help in waste biodegradation, then the bacterial strain was added.

Chemicals

The authentic B_{12} and folate were obtained from Sigma Aldrich Company. All the other chemical used in the current work obtained from Merck and the solvents used were HPLC grade.

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Methods

Maintenance of microorganisms

The fungal strain used in the current was maintained on PDA with subsequent monthly regeneration and preserved at 4°C. The bacterial strain of *k. pneumonia* was maintained in nutrient agar medium with subsequent regeneration before each experiment.

Waste processing

The agriculture waste (wheat bran) was treated by alkaline hydrolysis with sodium hydroxide according to the methods described by [5-7]. The fermentation process was carried out in the standardized way as developed where, wheat bran (20 g wet weight) was inoculated with a spore suspension of the fungus strain followed by the used bacteria.

Ethyl Methanesulfonate (EMS) mutagenesis

Five ml of overnight bacterial cells were centrifuged at 8000 rpm for 5 min and the pellet of bacterial biomass was suspended in a phosphate buffer 0.1 Mand pH 7. The suspended bacterial cells were treated with EMS mutagen for 60 min and then suspension was diluted and spread to the surface of PDA medium contained. The antibiotic discs were then laid on the surface, the plates were incubated for 2 days at 37°C, and the antibiotic resistant mutants were picked up from the nearest area around the discs (inhibition zone) and transferred to PDA plates for further studies.

Selection of mutants resistant to some antibiotics

Initial antibiotic sensitivity test (Kirby-Bauer,1979) was performed on wild type strain using different antibiotic discs (Oxoid Chemical Co., England) ($\mu g.disc^{-1}$) (Neomycin, 30; Tetracycline, 30; Chloramphenicol, 10; Ampicillin/Sulbactam20; Norfloxacin 10). The most sensitive antibiotics discs were used for selection of resistant mutants.

Isolation of total DNA from bacterial strains

Total DNA was isolated according to i-genomic BYF DNA extraction Mini Kit, iNtRON Biotechnology Inc., South Korea. The quantity and purity of the obtained DNA were determined according to the ultraviolet (UV)-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240).

RAPD analysis of the bacterial strains

For Polymerase chain reaction (PCR) technique, (PCR)-GOLD Master-Mix Beads (BIORON, Germany, Cat. No. 10020-96) were used. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Three different primers were used in the present study (Table1). All primers were supplied by Operon Technologies Company, Netherlands. To each ready-to-go PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: denaturation at 95°C for 5 min. 35 cycles each consists of the following steps: denaturation at 95°C for 1 min; primer annealing temperature for 2 min according to guaninecytosine (GC) ratio of each primer and incubation at 72°C for 2 min, for DNA polymerization. At the end, the PCR was held at 4°C till analysis. The amplified DNA products from RAPD analysis were electrophorated in 1% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 h. The different band sizes were determined against 100 bp ladder and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using both Polaroid Instant Camera and UV Transilluminator.

TABLE 1. The nucleotide sequence of the three primers used to evaluate the RAPD for different bacterial strains.

Primer code	5'→3' oligonucleotide sequence
P1	5′-CAT ACC CCC GCC GTT-3'
P2	5′-GTG TTG TGG TCC ACT-3'
P3	5′-TGA GTG GTC TAC GTG-3'

Fermentation process

Erlenmeyer flasks (250 ml) each containing sterile (20 g of wheat bran) solid agriculture substrate and 100ml sterile distilled water.Which previously inoculated with 1 X 10 ^{6} spores of the fungus /ml and incubated for 24 hr followed by the addition of $4.5X10^{8}$ bacterial cell/ml.The flasks were incubated statistically at $30C^{0}$ for

three days [8-10].

The flasks contents filtered to separate the culture growth and substrate from the culture media the filtrate was centrifuged at 4000 rpm for 10 min. The substrate consumed was also determined after each experiment by calculating the percentage of the difference of weight before and after the experiment. The contents of vit.B_{12} and folic acid were determined.

Estimation of vitamin B_{12} and folic acid

HPLC analysis was used for the simultaneous quantitative determination of vitamin B_{12} and folic acid in comparison with the standard according to the methods described by [11,12].

Results and Discussion

Antibiotic responses of Klebsiella penumonia

In order to isolate mutant strains producing vitamin B_{12} and folic acid higher than the wild type, mutagenesis treatment was performed by EMS. Firstly, the wild type was tested for antibiotics responsesusing Disc Diffusion Method (Kirby-Bauer technique). The results are shown in Table 2 where,the wild type strain was found to be sensitive towards all tested antibiotics with maximum sensitivity towards Tetracycline followed by Norfloxacin, Chloramphenicol, Streptomycin and Neomycin in descending order.

TABLE 2. Antibiotic sensitivity of *K. pneumonia* towards some antibiotics.

Antibacterial agent	Code	Disc potency(µg.disc^{-1})	Inhibition zone(mm)
Norfloxacin	NOR	10	25
Chloramphenicol	C	10	22
Tetracycline	TE	30	27
Neomycin	N	30	16
Streptomycin	S	10	18

Selection of the highly potent bacterial isolate

In the present experiments, the ability of (15 isolates) were tested for the production of vitamin B_{12} and folate. The results presented in Table 3 showed that all the tested mutants are B_{12} and folate producers, but they varied in their productivity. The best yields of vitamin B_{12} and folate (77.45,88.32ug/ml), respectively were obtained by isolate no(M33TE**).** Among the screened mutants only no. 9 and 5 produced vitamin B_{12} and folic acid higher than the wild type.

Generally, the mutations are obtained by the use of agents that interact with the deoxyribonucleic acid (DNA). So, the variability in the nucleotide sequence of DNA is basically detected by mutations. The induced mutation depends on the type of change in DNA sequence (base pair substitution, insertion, deletion etc.). Mutations are induced randomly in a microbial DNA by the application of chemical and physical mutagenic agents. Among the most popular mutagens used for the bacterial strain improvement is EMS which typically produces a variety of point mutations in the bacterial DNA. Through these point mutations, the different mutants are produced at high level in comparison of the natural mutations. Mutagenesis

TABLE 3. Screening for the potent K. *pneumoniae* isolate on the production of vit. B_{12} and folate.

Strain No	Vitamin B_{12} contents (µg / ml)	M/W %	Folic acid (µg /ml)	M/W %	Consumed Substrate (mg)	Specific B_{12} prod. µg/mg waste
Wild Type	43.56	-	55.21	-	1332	0.032
EMS-M32TE	45.99	105.5	66.21	121,4	1923	0.023
EMS-M33TE	77.45	178.4	88.32	159.9	2453	0.031
EMS-M34C	56.98	130.8	34.44	62.3	2432	0.022
EMS-M35C	44.56	102.2	56.21	101,8	1456	0.030
EMS-M36TE	67.43	154.7	87.56	158.8	2314	0.029
EMS-M37C	55.34	127.0	59.33	107.4	2234	0.026
EMS-M38NOR	23.43	53.7	45.342	82.12	2398	0.009
EMS-M39NOR	45.56	104.5	56.34	102	2432	0.021
EMS-M310NOR	33.99	78.0	66.43	120	1786	0.014
EMS-M311S	28.34	65.0	44.34	80.3	1654	0.021
EMS-M312S	33.76	77.4	56.32	102.0	1983	0.017
EMS-M313S	34.44	79.06	43.32	78.4	1231	0.027
EMS-M314N	55.21	126.7	33.34	60.3	2298	0.025
EMS-M315N	44.21	101.4	54.33	98.4	2156	0.024

M/W % mutant to the wild strain percent.

of industrial microbial strains is widely used for the enhancement of the microbial productivity [13-15].

The results obtained after EMS-mutagenesis were in agreement with those obtained b.[16- 19]. They obtained an eight fold increase in folic acid by *B.subtilis* after applied a targeted mutagenesis method. Random mutagenesis and exposure to the folate antagonist methotrexate were both shown to be successful in obtaining folateoverproducing strains of *Lb. plantarum*, although this phenotype was shown to be rather unstable [20]. A common method to improve vitamin B_{12} yields is random mutagenesis and the use of genetic engineering [21-23]. Mutations in this regulatory region result in riboflavin overproduction [24-27].

Different carbon sources

In the present experiment the effect of different carbon sources (glucose, Fructose, Mannose, Sucrose, Maltose, lactose, starch and Dextrin) on the production of vit. B_{12} and foalte were investigated. The results presented in Table 4 showed that the best B_{12} and folate outputs were (267 and 121 ug/ml), respectively. These results correlated with that stated by [8,10] since, mono-sacchariedes specially glucose preferred by the microbial cells for energy production and utilization**.**

TABLE 4. Effect of different carbon source on the production of vitamin B_{12} and folate using *K. pneumoniae* EMS-M33TE.

Carbon Source	Vitamin B_{12} contents (µg /ml)	Folic acid (µg /ml)	Consumed substrate (mg)	Specific vit.B_{12} productivity µg/mg waste
Control	77.43	88.32	2453	0.031
Glucose	267.34	121.43	2543	0.104
Fructose	213.67	139.99	2567	0.082
Mannose	167.80	110.12	2145	0.077
Sucrose	211.43	87.76	2232	0.094
Maltose	145.88	57.87	2675	0.054
Lactose	165.91	66.99	2598	0.063
Starch	122.21	77.41	2329	0.052
Dextrin	120.64	76.90	2143	0.055

Control. without addition of carbon source.

Glucose concentration

Different glucose concentrations (2,4,6,8, 10,12, and 15g/flask) were investigated for the production of vit. B_{12} and folate. The results presented in Table 5 showed that the best B_{12} and folate output (356.9,278.22ug/ml) was obtained at the addition of 10 g glucose to the fermentation medium. At the lower concentrations (2,4,6,8 g/flask) remarkable increase in the yields of B_{12} and folate . On the other hand, at the higher concentration (12,14,15g/flask), a considerable decrease in the products outputs, this may be due to

TABLE 5. Effect of different glucose concentration on the production of vit.B_{12} and folate *K. pneumonia* EMS-M33TE.

Glucose concentration g/flask	Vitamin B_{12} contents (ug/ ml)	Folic acid (ug/ml)	Consumed substrate Mg	Specific vit. B_{12} productivity µg/mg waste
0 (control)	77.40	88.32	2453	0.031
2	100.32	123.22	2975	0.033
4	145.36	156.44	2765	0.052
6	245.66	245.89	2452	0.088
8	345.25	221.67	2647	0.130
10	356.95	278.22	2431	0.146
12	221.62	299.42	2139	0.103
14	234.70	241.11	2298	0.102
15	225.52	213.34	2299	0.098

Control .without addition.

substrate toxicity as a results of high concentration. The data presented in Table 5 revealed that the best B_{12} and folate yields (356.95, 278.3 ug/ml), respectively. At the low concentrations a gradual increase in the production of vitamin B_{12} and folate were noticed.

Molecular Analysis of Some Superior Mutants

The DNA nucleotide sequence of the superior mutants compared to the wild type strains strain was evaluated to detect any genetic effect of UV-mutagenesis on the DNA. Three random primers obtained from Operon Technologies were used to identify the genetic variability among the four superior mutants all these primers were successfully amplified.

Specific fragments of the genomic DNA. The RAPD technique was used in order to test if genetic markers could be correlated with the production of vitamin B_{12} and folic acid. The application of the three random primers (Fig. 1) with the genomic DNA of the wild type strain (No.1) and two superior mutants clearly noticed that four amplified bands with primer 1 were occurred when DNA of the wild type strain (lane 1) was used as a template. Also, the same four amplified bands were occurred when DNA of the two mutants (lanes 2 and 3) were used as a template in addition of one band for each mutant. In the lane 2 of the mutant No.**EMS**-60-M110, a new distinct band with size of 400 bp was detected. Moreover, in the lane 3 of the mutant No.**EMS**-60-M110, a new distinct band with size of 3000 bp was obtained. When used the primer 2, the band number of the wild type strain (No.1) was one (1500 bp) while the band number of the superior mutants were 1500 and 1200 bp (lanes 2 and 3). Also, the application of the primer 3 produced one band (300 bp) with the wild type strain (No.1) while the band number of the superior mutant (lane 2) were 300, 1350 and 3000 bp and the band number of the superior mutant (lane 3) were 1350 and 3000 bp.

On the other hand, the application of primer No.1 (Fig. 2) with the genomic DNA of the wild type strain (No.3) and two superior mutants clearly noticed that five amplified bands occurred when DNA of the wild type strain (lane1) were used as a template. The same five amplified bands occurred when DNA of the superior mutant (lanes 3) were used as a template but three bands only obtained with the mutant (lane 2). Moreover, the amplification reactions with the primer No.2 (Fig. 3) generated three bands with the wild type strain (No.3). The same three amplified bands occurred when DNA of the two superior mutants were used

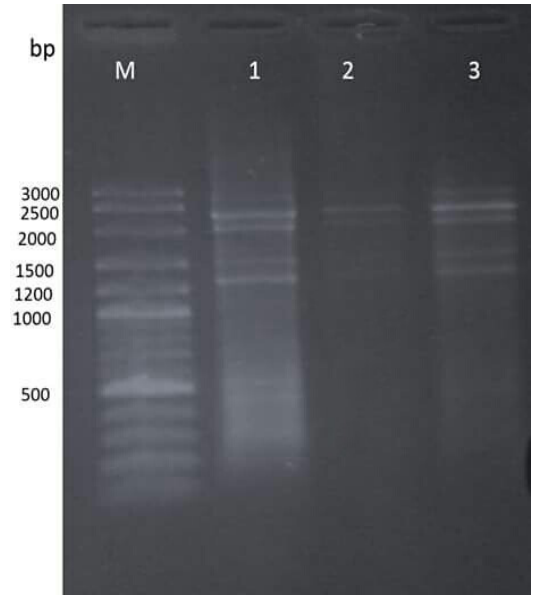

Fig. 1. Photograph of DNA amplified banding patterns based on RAPD for two different mutants: M3 (lane 2) and M 6 (lane 3) in comparison with *K. pneumonia* (lane 1) using primer (P1)against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M).

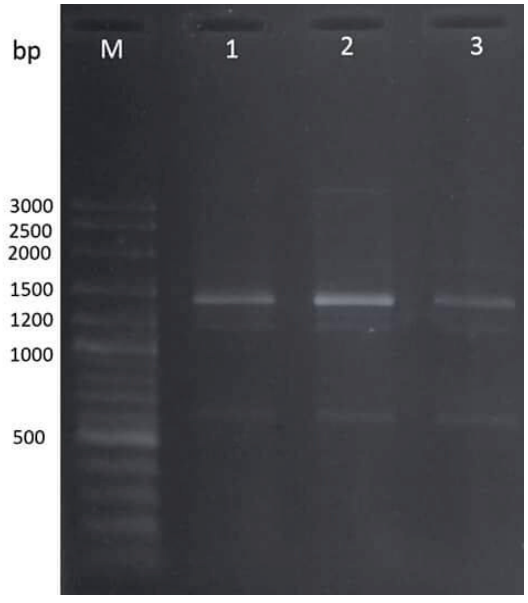

Fig. 2. Photograph of DNA amplified banding patterns based on RAPD for two different mutants: M3 (lane 2) and M6 (lane 3) in comparison with *K. pneumonia* (lane 1) using primer (P2)against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M).

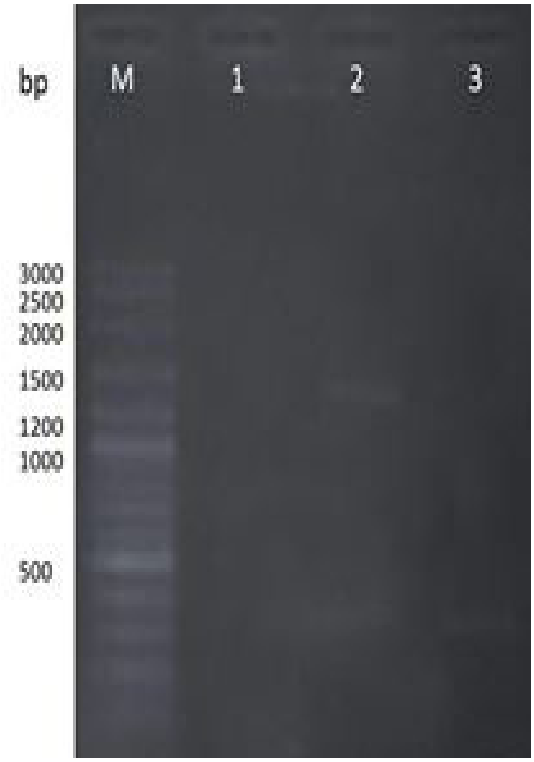

Fig. 3. Photograph of DNA amplified banding patterns based on RAPD for two different mutants: M3 (lane 2) and M6 (lane 3) in comparison with *K.pneumonia* (lane 1) using primer (P3) against VC100 bp plus DNA ladder Vivantis # NL 1407- Malaysia (lane M).

as a template but other bands (above 3000 bp) only obtained with the mutant (lane 2). Finally, the application of the primer 3 did not produce any band with the wild type strain (No.3) while the band number of the superior mutant (lane 2) were 300 and 1350 bp and the band number of the superior mutant (lane 3) was 300 bp.

The above variability in RAPD patterns confirmed the evidence of changes in *K.pneumonia* genome after EMS-mutagenesis. Furthermore, some of these differences based on RAPD technique, could be used as genetic markers for screening of the new induced mutants after EMS-mutagenesis. The obtained results are in agreement with those reported by [28,29,30] who demonstrated that some differences and similarities in RAPD profile between mutants in lactic acid bacteria obtained after UV and EMS-mutagenesis. Moreover, [24] detected many differences in mutant and fusant strains in comparison with the wild type strain of *S.cerevisiae*, some differences in mutant strains in comparison with the original strain [31,32]. These differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *K.pneumonia* genome after EMS-mutagenesis.

Conclusion

The present study gave insight about the improvement of B_{12} and folate production using the normal mutagenesis using EMS. The results showed that all the selected mutants are vitamin producers and the best isolate was *K. pneumonia* EMSM33ET. On the other hand, the addition of glucose appeared to improve the productivity by about three fold compared to the wild strain when added to the solid substrate which will be tacking in the future application and studies. PCR studies revealed that clear differences at bands number and size between the original strain and its mutants using RAPD analysis by three different random primers. These differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *K. pneumonia* genome after EMS-mutagenesis.

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انتاج فيتامين ب ١٢ وحمض الفوليك باستخدام طفرات من بكتيره كلبسيلا بنيمونيا

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[٢] قسم الوراثه والسيتولوجى - شعبه الهندسه الوراثيه - المركز القومى للبحوث - القاهرة - مصر.

تم دراسه انتاج فيتامين ب 12 وحمض الفوليك باستخدام تقنيه التخمير الصلب بواسطه بعض الطفرات من بكتيره كلبسيلا بنيمونيا . أشارت النتائج أن العزله البكتيريه رقم EM33TE اعطت أفضل انتاجيه لكل من فيتامين ب 12 وحمض الفوليك (77,45 , 88,22 ميكروجرام /ملى بالتناوب.

أمتدت الدراسه الى استخدام بعض المصادر الكربونيه المختلفه (جلكوز, مانوز,سكروز, مالتوز , لاكتوز , نشا ودكسترين) حيث اشارت النتائج ان أفضل انتاجيه 356,9 ميكروجرام/ ملى من خلال اضافه الجلوكوز بنسبه 10جم/ لتر.

كشفت الدراسات الجزيئية بإستخدام جهاز تفاعل البلمرة المتسلسل أن الاختلافات كانت واضحة بين السلالة الأصلية والطفرات الناتجة منها باستخدام تحليل رابد بإستخدام ثلاثة بريمر عشوائية وهذه الاختلافات الجزيئية بإستخدام جهاز تفاعل البلمرة المتسلسل تعتبردليل على الاختلافات الجينية بين الطفرات و جينوم كالبسيلا بينمونيا بعد إستحداث الطفرات بإستخدام المطفر الكيميائي الإيثيل ميثان سالفونيت.