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Selection of Microorganisms Producing Vitamin B_{12} from Local (Saudi) Isolates

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ABSTRACT. Forty-six bacterial strains were highly varied in their efficiency to produce cell biomass and vitamin B_{12}. *Propionibacterium freudenreichii* ATCC 6207 and two local bacterial isolates (*Streptomyces* S184 and *Methylomonas methanica* 14B-16 were the most efficient strains. Productivity of vitamin B_{12} was affected by culturing methods (shake and static), and growth phases. The highest Vitamin B_{12} productivity was observed in shake culture of *P. freudenreichii* (170.2 μg/100 ml culture and 68.1 μg1/100 mg biomass dry wt.). This strain also showed a positive correlation between the amount of vitamin and elapsing of time. Other bacterial strains gave amounts of Vit. B_{12} ranging from 9.12 to 35.6 μg/100 ml culture and from 1.19 to 43.6 μg/100 ml dry wt. biomass. The most efficient strains also showed a high productivity at early stage of growth in shake culture.

Introduction

Vitamin B_{12} (water-soluble vitamin) is very important for human and many domestic animals. It controls pernicious anaemia in humans (antipernicious anaemia factor). Vitamin B_{12} is produced chiefly by actinomycetes and bacteria[1] Some species of *Propionibacterium* are considered to be the best microorganism for production of vitamin B_{12}[2]. The biosynthesis of this vitamin by *Streptomyces aureofaciens, Escherichia Coli, Pseudomonas dentrificans, Clostridium sticklandii, Corynebacterium* sp. *Propionibacterium freudenreichii, Eubacterium limosum* and *Bacillus megaterium* was studied by many investigators[3-7].

This study was carried out to select the most efficient strains for production of vitamin B_{12} from local (Saudi) isolates. Productivity of vitamin B_{12} and growth parameters were also investigated during different microbial growth phases.

Material and Methods

Microorganisms used

In this investigation forty-four strains of *Streptomyces*[8] and one strain of *Methylomonas methanica* 14B-16[9] were obtained from the department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arábia (local isolates). Bacterial strains of *Propionibacterium freudenreichii* (6207) was obtained from American Type Culture Collection (ATCC). *Streptomyces* strains, *M. methanica* 14B-16 and *P. freudenreichii* were subcultured on malt extract agar[10], methanol mineral medium[11] and tomato juice peptone and yeast extract agar medium[10] respectively and maintained at 5°C.

Selection of the most efficient strains producing B_{12}

Conical flasks (250 ml in volume) containing 50 ml malt extract medium[10] were used for the propagation of *Streptomyces* strains and *P. freudenreichii* (6207). The similar flasks containig methanol mineral medium were used for cultivation of *M. methanica* 14B-16. The flasks were inoculated with 1 ml of bacterial suspension (10^3-10^4 conidiospores/ml in case of *Streptomyces* strains and 10^4-10^5 cells/ml for other bacteria). The inoculated flasks were shaked on reciprocal shaker (120 stroke/min) at 28°C for 10 days. Cultures were used at the end of incubation to determine the microbial biomass, consumed sugar and vitamin B_{12} content.

Growth curves for selected strains

Conical flasks containing broth media were inoculated with the selected strains including *Streptomyces* S184, *P. freudenreichii* (6207) and *M. methanica* 14B-16. These bacteria were described before. The first group was shaked using reciprocal shaking incubator (120 stroke/min), whereas the second static culture was left without shaking. The flasks were then incubated at 28°C for 8-10 days. Three flasks of each strain were taken daily from static and also from shaken cultures to determine the microbial biomass, consumed sugar, and growth parameters.

Growth parameters

Microbial growth parameters were calculated according to Doelle[12]. These parameters were specific growth rate (u), doubling time (t_d), generation number (n) and multiplication rate (mr).

Biomass determination

Microbial biomass was determined by centrifugation of certain volume of the culture at 4000 to 5000 rpm. The sediment was washed twice and resuspended in distilled water. This microbial suspension was divided into two equal parts, one of them was filtered through millipore membrane and dried at 70°C for 3 days while the other was used for the determination of vitamin B_{12}.

Chemical determinations

Vitamin B_{12}. Vitamin B_{12} was expected and determined colorimetrically in the microbial biomass at 550 nm using the method described by Fredrick[13].

Sugar consumed. Sugar content of the cultures (supernatant) was determined according to Dubous *et al.*[14].

Statistical analysis. Statistical analyses were computed according to Steel and Torrie[15] using C.B.M. computer.

Results and Discussion

Selection of microorganisms

In this investigation 44 strains of *Streptomyces* sp, *Propionibacterium freudenreichii* (6207) and *Methylomonas methanica* 14B-16 were tested for their efficiency to produce vitamin B_{12}. Results in Table 1 show that the amount of microbial biomass and vitamin B_{12} varied highly from one strain to another. The highest biomass was obtained in case of *Streptomyces* S28, *Propionibacterium freudenreichii* ATCC

TABLE 1. Biomass and B_{12} in shaken culture of different bacterial strains

Bacterial strains	Dry wt. g/100 ml	B_{12}		Bacterial strains	Dry wt. g/100 ml	B_{12}	
		mg/L*	µg/100 mg**			mg/L*	µg/100 mg**
Strept. S1	0.670	0.342	5.1	*Strept.* S173	0.480	0.254	5.3
„ S3	0.250	0.402	16.1	„ S174	0.400	0.318	8.0
„ S4	0.500	0.328	6.8	„ B177	0.285	0.517	18.1
„ S7	0.470	0.240	5.1	„ S180	0.346	0.404	11.7
„ S9	0.720	0.258	3.6	„ S184	0.276	0.976	35.4
„ S11	0.680	0.377	5.5	„ A189	0.382	0.583	15.3
„ S12	0.510	0.328	6.6	„ A192	0.202	0.347	18.0
„ B19	0.160	0.230	18.1	„ S193	0.200	0.274	13.7
„ S27	0.640	0.430	6.8	„ S195	0.370	0.296	8.0
„ S28	1.300	0.385	2.5	„ S197	0.490	0.502	10.2
„ S38	0.655	0.565	8.6	„ S199	0.340	0.412	12.1
„ B44	0.700	0.380	5.4	„ S200	0.180	0.256	14.2
„ S51	0.670	0.265	4.0	„ A208	0.189	0.158	8.9
„ B57	0.380	0.300	7.9	„ B220	0.264	0.389	16.0
„ B74	0.180	0.271	15.1	„ B233	0.440	0.574	13.0
„ S86	0.190	0.281	14.8	„ B251	0.189	0.388	20.5
„ S105	0.400	0.295	7.4	„ B252	0.629	0.279	24.5
„ S125	0.530	0.312	5.9	„ S253	0.106	0.261	24.7
„ B139	0.240	0.563	23.5	„ S254	0.253	0.348	10.4
„ B141	0.580	0.334	5.8	„ S262	0.729	0.512	7.1
„ B157	0.530	0.277	5.2	„ B275	0.400	0.308	7.8
„ B169	0.440	0.348	7.9	*M. methanica*	0.191	0.629	32.9
„ S171	0.480	0.254	5.3	*P. frender.*	1.130	1.424	162.0

*mg vit. B_1 culture **µg vit. B_{12}/100 mg dried biomass.

(6207), *Streptomyces* S262 *Streptomyces* S9, and *Streptomyces* B44. The dry weight of their biomass was 1.300, 1.130, 0.729, 0.720 and 0.700 g/100 ml culture respectively. On the contrary, *Streptomyces* B19 and *Streptomyces* S253, gave the lowest biomass being 0.160 and 0.106 dry wt/100 ml culture respectively. The most efficient strains in the production of vitamin B_{12} were *P. freudenreichii* ATCC 6207, *Streptomyces* S184 and *M. methanica* 14B-16 where they produced 1.424, 0.976 and 0.629 mg vitamin B_{12}/L culture respectively. The corresponding observed values for vitamin B_{12} in the biomass were 162,35.4 and 32.9 μg/100 mg dry weight biomass respectively. The lowest efficiency in the production of vitamin B_{12} was noticed in the cultures of *Streptomyces* (A208) being 0.159 mg/100 ml culture (8.9 μg/100 mg dry weight).

It is clear from this result that the best strains for production of vitamin B_{12} were three out of 46 tested microorganisms. These organisms were *P. freudenreichii* ATCC 2607, *Streptomyces* S184 and *M. methanica* 14B-16. These results are in agreement with Florent and Ninet[16] who stated that some methanol assimilating bacteria such as *Methanobacterium omelianski* and *Protoaminobacter ruber* could produce vitamin B_{12}. The cultures of both bacteria contained 250 and 880 μg vitamin B_{12}/100 ml culture. It was also found by some authors that the yield of vitamin B_{12} in *P. freudenreichii* reached 10.4 μg/100 mg wet cells and 3.74 mg/100 ml cultures[17].

Growth curves

P. freudenreichii ATCC 6207 grew exponentially during the first 2 days in shake cultures and the first 4 days in static cultures (Fig. 1) where the maximum growth was

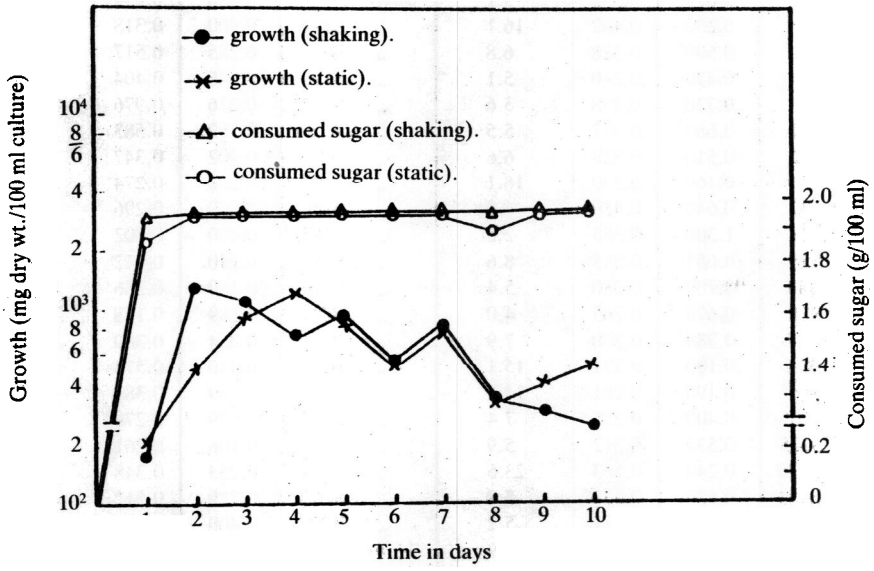

FIG. Growth curves and consumed sugar in shake and static culture of *Propionibacterium freudenreichii* ATCC 6207.

1.39 and 1.21 g dry wt/100 ml culture respectively. Specific growth rate of this bacterium was higher in shake cultures than static cultures being $0.085h^{-1}$ and $0.025h^{-1}$ respectively. Number of generations, multiplication rate and hourly growth rate also showed the same trend. On the contrary, the lowest value of doubling time (t_d) was recorded in shake cultures being 8.15 hours (as compared with 27.73h for static culture). After the exponential growth phase in both types of cultures, the growth density slightly decreased to be more constant up to the end of incubation time. Sugar utilization efficiency (amount of consumed sugar per unit of original sugar) reached its maximum (0.989) on the 2*nd* and 3 *rd* days of incubation for shake and static cultures respectively. The vitamin content of biomass (shake flasks) increased significantly during 10 days incubation period, being 32.7 and 68.1 μg/100 mg dry wt. on 9*th* and 10*th* days of incubation respectively (Fig. 2). The corresponding figures of vitamin content in the culture were 96.4 and 170.2 μg/100 ml culture. Static cultures showed a lower content of this vitamin as compared with shake cultures (6 μg/100 mg dry wt. and 30.5 μg/100 ml culture on the 10*th* day of incubation).

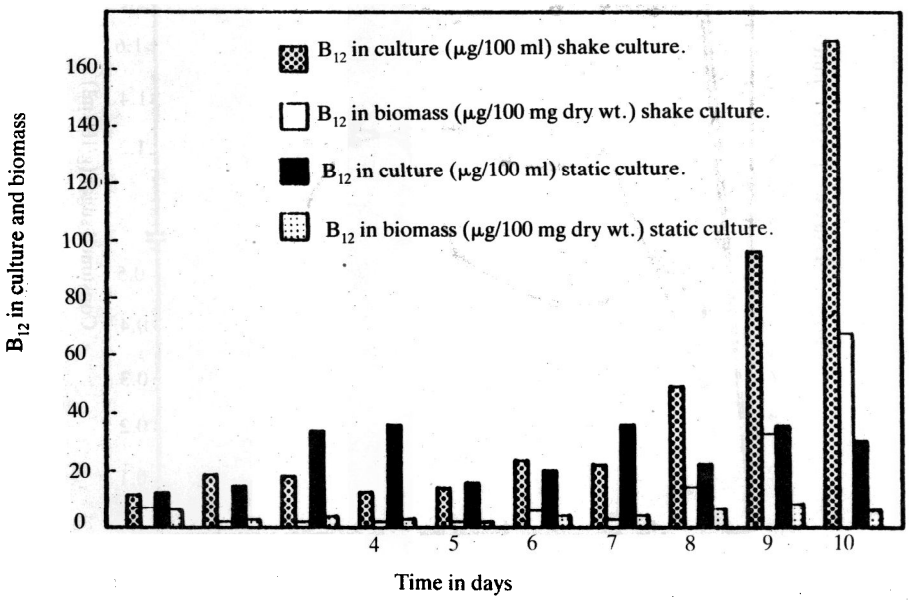

FIG. 2. Vitamin B_{12} in biomass and culture of *Propionibacterium freudenreichii* ATCC 6207 (shake and static cultures).

Streptomyces S184 showed early exponential phase (3 days of incubation) in shake cultures and late exponential growth (5-7 days) in static culture (Fig. 3). The growth at the end of this phase was 910.0 and 1050.0 mg dry wt/100 ml culture respectively. The corresponding figures for specific growth rate were 0.059 and $0.36h^{-1}$ respectively. Sugar utilization efficiency (0.903 and 0.880 for shake and static cultures re-

spectively) thereafter the values became stable and ranged from 0.835 to 0.962. Results also depicted that the amount of vitamin B_{12} in the culture of shake flasks increased gradually with the increase of time where 9.74, 19.79, 25.21 and 33.09 μg vitamin/100 ml culture were observed after 1, 4, 8 and 9 days of incubation respectively (Fig. 4). In contrast, static flasks showed low amount of vitamin and did not exhibit the same trend of shake flasks, where the corresponding figures were 9.59, 10.52, 13.61 and 11.75 μg/100 ml culture.

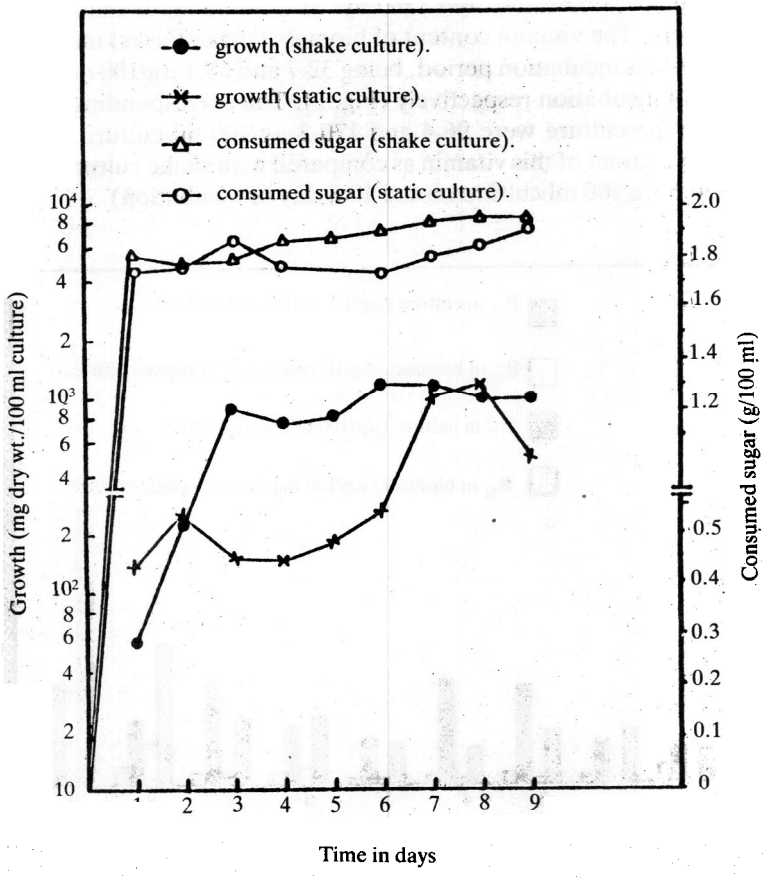

FIG. 3. Growth curves and consumed sugar in shake and static cultures of *Streptomyces* S184.

Both culturing methods of *Methylomonas methanica* 14B-16 showed 2 days lag period followed by a gradual increase in biomass (exponential phase) with 0.0187 and 0.0269h^{-1} specific growth rate for static and shake cultures respectively (Fig. 5). The biomass productivity at the end of exponential phase was 230 and 255 mg/100 ml culture respectively. Thereafter, the growth rate decreased gradually (phase of decelerating growth) entering the stationary phase at the last 2-3 days of incubation.

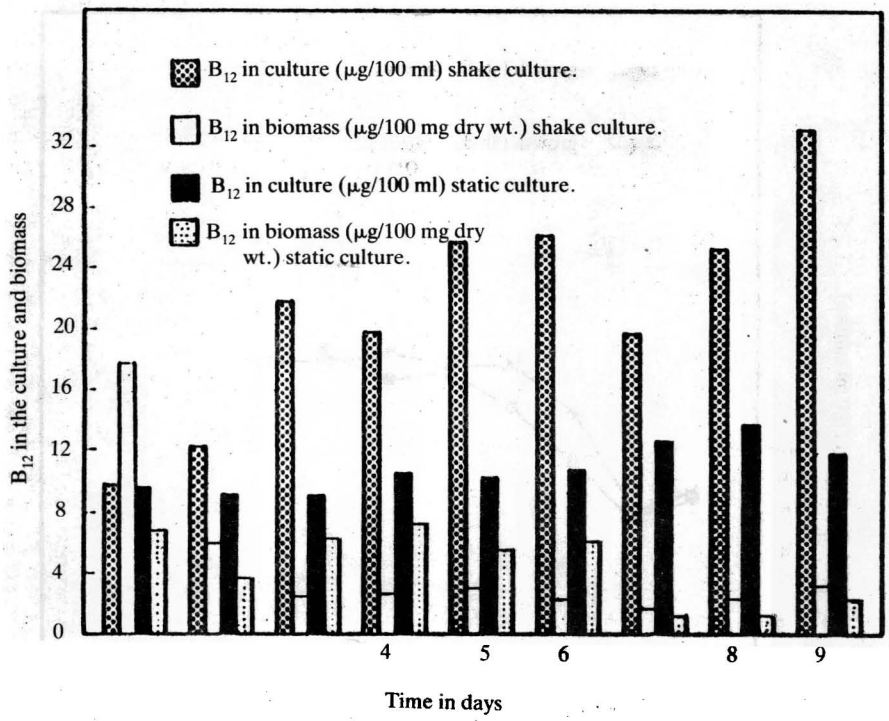

FIG. 4. Vitamin B_{12} in biomass and culture of *Streptomyces* S184 (shake and static cult

The maximum productivity of this vitamin in culture was recorded after 3 days in shake cultures and 5-7 days in static cultures being 35.6 and 17.1 to 17.5 μg/100 ml culture respectively (Fig. 6). The same trend was also observed in vitamin content of biomass where 43.6 and 18.1 μg vitamin B_{12}/100 mg dry wt. were found in the biomass of shake and static cultures respectively on the 2*nd* day of incubation. The amount of vitamin during other days of incubation fluctuated from 9.5 to 39.5 μg/100 mg biomass in shake cultures and from 5.6 to 16.6 μg/100 mg biomass in static cultures.

It is clear from these results that the three tested bacteria showed a wide variation in their growth rates, biomass and vitamin B_{12} productivity. Culturing methods (shake and static cultures) and incubation time had a clear effect on these bacteria and this effect highly varied from one strain to another.

Data regression analysis showed that a high positive correlation coefficient between biomass of *Streptomyces* S184, *M. methanica* 14B-16 and incubation period. It means that the increase of incubation period led to an increase in productivity of biomass and this increase was more pronounced in shake culture than static one ($R = 0.834$ and 0.969 for shake cultures of *Streptomyces* S184 and *M. methanica* 14B-16 respectively, whereas the corresponding figures for static cultures were 0.709 and

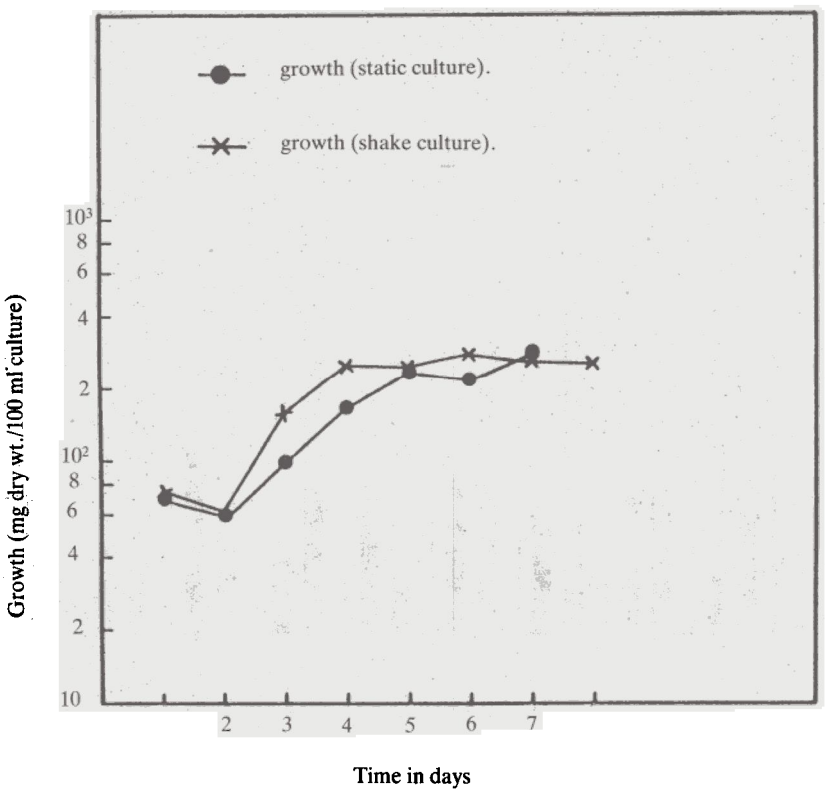

FIG. 5. Growth curves of *Methylomonas methanica* 14B-16 in shake and static cultures.

0.908). On the contrary, *P. freudenreichii* ATCC 6207 showed a negative correlation coefficient between growth and incubation period. It means that this organism forms the bulk of its biomass during the first 1-2 days of incubation (see Fig. 2). The highest correlation coefficient between consumed sugar and elapsing of time was recorded in case of *Streptomyces* S184 grown in shake culture, being 0.943.

Although *P. freudenreichii* showed a negative correlation coefficient between the biomass and incubation period in both culturing methods, it gave a positive correlation value between vitamin B_{12} content of biomass and elapsing of time as compared to other bacterial strains, which imply that elapsing of incubation period led to an increase in biosynthesis of vitamin B_{12} in the biomass of *P. freudenreichii* ATCC 6207.

Generally, it could be concluded that the biomass and vitamin B_{12} productivity of *P. freudenreichii* ATCC 6207 were observed in high values as compared with other bacterial strains (*Streptomyces* S184 and *M. methanica* 14B-16). Strains of *Propionibacterium freudenreichii* and *Propionibacterium shermanii* were used by some investigators as best organisms for production of vitamin B_{12} (3.74 μg/1 ml culture and

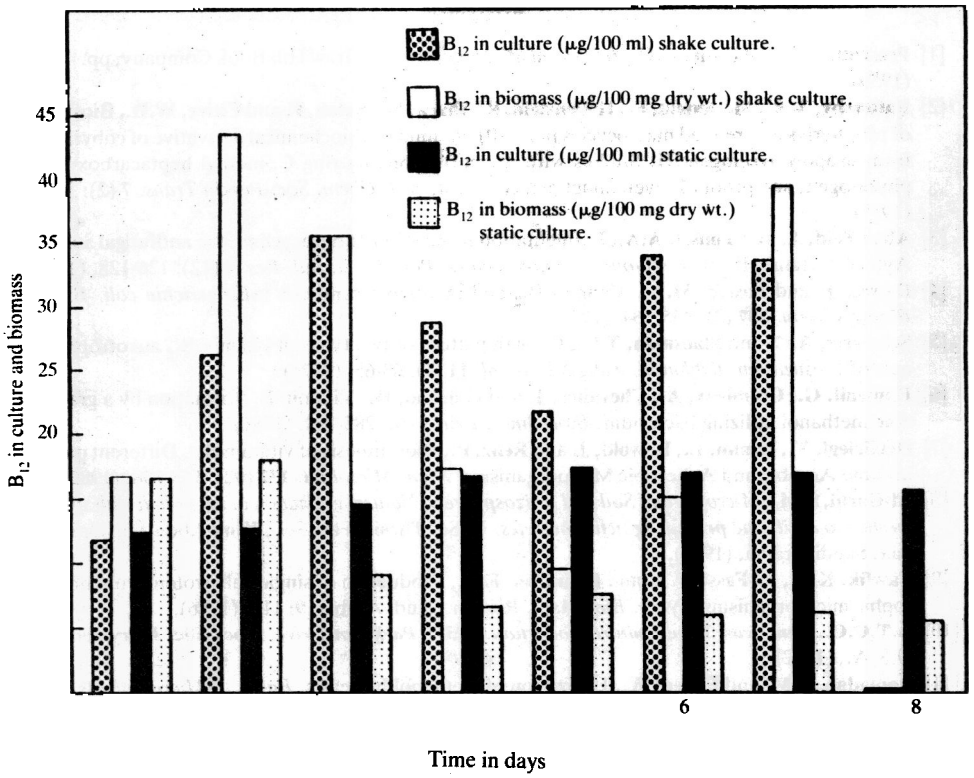

FIG. 6. Vitamin B_{12} in biomass and culture of *Methylomonas methanica* 14B-16 (shake and static cultures).

7.11 μg/100 mg cells respect.)[17-18]. Hoerig and Renz[19] and Lamm *et el.*[20] also reported that the use of malt extract stimulated the biosynthesis of vitamin B_{12} due to the presence of vitamin B_{12} (riboflavin) which plays an important role in the biosynthesis of 5, 6 dimethyl benzimidazole (precurser for the biosynthesis of vit. B_{12}). A strain of facultative methylotrophic bacteria (EMO2T) gave 260 μg vitamin B_{12}/100 ml culture during its growth on methanol as sole carbon sourse[21-22]. This amount was higher than that observed in this investigation (35.6 μg/100 ml culture). On the other hand, the local isolate of *Streptomyces* S184 showed higher vitamin B_{12} productivity than that observed by some investigators such as Sultanova and Schelkova[23] who mentioned that *Actinomyces adoratus, A. aromaticus, Streptomyces anulatus, S. griseus, S. globisporus, S. candidus* produced 0.14 to 0.9 μg/100 ml culture (25.9 ng/100 mg dried cells). In this investigation the actinomycetes local isolate (*Streptomyces* S184) gave 9.74 to 33.09 μg vitamin B_{12}/100 ml culture (1.2 to 17.71 μg/100 mg dry wt). This finding confirms the results obtained by Ledingham[21] who stated that *S. griseus* and *S. Olivaceous* produced 3¼ μg vit. B_{12}/100 ml culture. This variation in vit. B_{12} productivity by microorganisms may be attributed to many factors such as culturing method, incubation period, media used and bacterial strains.

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انتقاء الكائنات الحية الدقيقة المنتجة لفيتامين ب$_{١٢}$ من عزلات محلية (السعودية)

منصور جميل سجيني ، الشحات محمد رمضان و عبد الرحمن محمد القرشي
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المستخلص . أظهرت ست وأربعون سلالة من البكتريا تنوعا كبيرا في كفايتها لإنتاج الكتلة الحيوية وفيتامين ب$_{١٢}$. وكانت بكتريا البروبيونيباكتيرم فريدنريشياي ٦٢٠٧ وعزلتان محليتان من البكتريا (ستربتوميسس إس ١٨٤ ، ميثايلوموناس ميثانيكا) من أكفأ سلالات البكتريا المختبرة . وتأثرت الإنتاجية من فيتامين ب$_{١٢}$ بطرق التنمية (مزارع مهتزة وساكنة) ومراحل النمو . ولوحظت أعلى إنتاجية من فيتامين ب$_{١٢}$ في المزارع المهتزة لميكروب البروبيونيباكتيرم فريدنريشياي (١٧٠,٢ ميكروجرام/١٠٠ مل مزرعة ، ٦٨,١ ميكروجرام/١٠٠ مجم كتلة حيوية جافة) وقد أظهرت هذه السلالة أيضًا معامل ارتباط موجبًا وقويًا بين كمية الفيتامين المتكونة وفترة التحضين . وأعطت سلالات البكتريا الأخرى كميات من فيتامين ب$_{١٢}$ تراوحت ما بين ٩,١٢ إلى ٣٥,٦ ميكروجرام/١٠٠ مل مزرعة ، ١,١٩ إلى ٤,٦ ميكروجرام/١٠٠ مجم كتلة حيوية جافة .