

Selection of Methylophilic Microorganisms for the Formation of Single Cell Protein

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ABSTRACT. Methanol-assimilating microorganisms were isolated from 27 soil samples. Thirty-one (26 bacteria and 5 yeasts) out of 385 isolates were found to be methylophilic. Fungal and actinomycetes isolates did not grow on methanol as a sole carbon source. The growth parameters of yeast and bacterial isolates in shake flasks cultures widely varied from one isolate to another. The methylophilic bacterial strains 14B-16, 11B-11, 01B-01 and 10B-11 showed the highest specific growth rates, being 0.083, 0.059, 0.058 and 0.057 h⁻¹ respectively. *Pichia* sp. 2y-17 gave a short exponential phase as compared with bacterial isolates. The methylophilic bacterial strain 14B-16 gave the highest biomass and protein productivity (113.30 and 73.88 mg/100 ml culture respectively). Effective yield of tested yeast was found to be of a low value. Microbial growth was highly affected by the high concentration of formaldehyde.

Introduction

Methanol is available in large quantities and high purity. It is directly produced from methane or methane-related raw materials. The use of methanol in the production of single-cell-protein (SCP) is of a great importance since this substrate is a common product of industrial operation. Anthony^[1] stated that the methylophilic microorganisms are those organisms able to grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds. A limited number of yeasts is known to assimilate methanol as facultative methylophilic such as *Pichia pastoris*, *Pichia pinus*, *Candida boidinii* and *Hansenula polymorpha*^[2,3]. Methylophilic bacteria include the obligate methylophilic such as *Methylomonas* and *Methylococcus* and facultative methylophilic such as *Methylobacterium*^[1].

Methylotrophic bacteria have the advantage over yeasts of higher yield coefficients, growth rates and protein content; and they are the organisms chosen by Imperial Chemical Industries (ICI United Kingdom), the Mitsubishi Gas Company (MGC Japan) and Norproteine (Scandinavia) as the basis for investigations and development of methanol-based SCP processes. On the contrary, yeasts have a number of general advantages over bacteria as a source of SCP, including their lower nucleic acid content and their greater (psychological) acceptability for human consumption. In addition, the lower pH for optimum growth is also an advantage in minimising bacterial contamination^[1,2 and 4].

The objective of this study was to study the efficiency of different methylotrophic microorganisms isolated from soils in the utilization of methanol as a sole carbon source. The growth parameters and protein content of these organisms were also elucidated.

Material and Methods

Soil and Samples

Microorganisms were isolated from soils. Sixteen soil samples were collected from area polluted with petroleum oil wastes at the north of Jeddah (Elrehaily Petroleum Station). Another eleven soil samples were obtained from cultivated soil of the western region of Saudi Arabia. The soil samples were transferred into plastic bags and were stored at 4°C in a refrigerator until microbiological analysis.

Isolation

A 30 gram representative sample of each soil was added to 100 ml methanol mineral medium in a 250 ml conical flasks. This medium contained 3 g $(\text{NH}_4)_2\text{SO}_4$, 2 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 1 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 mg Na_2MoO_4 , 0.1 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.03 mg H_3BO_3 and 4 g methanol/L distilled water and was adjusted at pH: 7.0^[3]. This medium was also supplemented with 50 mg yeast extract/L. The flasks were shaken on a reciprocal shaker (100 stroke/minute) for 10 days at 30°C. Thereafter one ml of each flask was transferred into a petri-dish containing agar medium and was spread by a sterile curved glass rod. Another three Petri-dishes were streaked immediately with the same glass rod. Petri-dishes were then incubated at 30°C for 2-7 days. Potato dextrose agar, malt extract agar, nutrient agar^[5] and starch casein agar^[6] media were used for the isolation of fungi, yeasts, bacteria and actinomycetes respectively. Methanol mineral medium^[7] was used for isolation of obligate methylotrophs.

Screening of methylotrophic microorganisms

The microbial cultures isolated from different soil samples were purified and tested for their ability to utilize methanol as a sole source of carbon. These cultures included 277, 43, 12 and 53 isolates from bacteria, actinomycetes, yeasts and fungi respectively. Conical flasks (100 ml in volume) containing 25 ml methanol mineral

medium (4 g methanol/100 ml) were inoculated with 1 ml of cells or spores suspension (O.D. = 0.20) of the tested organisms and were incubated at 30°C using a reciprocal shaker (100 stroke/min.). After 10 days incubation, the microbial growth was recorded.

Efficiency of methylotrophic microorganisms

The methylotrophic isolates (26 bacteria and 5 yeasts isolates) were used in this experiment. Conical flasks (250 ml cap.) containing 100 ml methanol mineral medium (4 g methanol/100 ml) were inoculated with 0.1 ml cells suspension of the tested organisms (containing 5.1×10^4 or 7.2×10^5 cells/ml for bacteria and yeasts respectively) grown on methanol mineral medium for 5 days. The flasks were shaken on a reciprocal shaker (100 stroke/min.) for 7-11 days at 30°C. Samples (5 ml) were taken from the cultures at 24 hours intervals to determine their optical density (O.D.) at 570 nm. The relationship between microbial growth and time was then plotted on semilogarithmic papers. Different growth parameters were calculated from the exponential phase of growth^[8 and 9] as follows:

1 – Specific growth rate (μ) = $\ln A - \ln A_0 / (t - t_0)$.

where:

$\ln A$ = the Napierian logarithm of growth (A) at a certain time (t) of exponential phase.

$\ln A_0$ = the Napierian logarithm of growth (A_0) at zero time (t_0) of exponential phase.

2 – Doubling time (t_d) = $\frac{\ln 2}{\mu}$

3 – Number of generations (N) = $\log A - \log A_0 / \log 2$

4 – Multiplication rate (MR) = N/time.

Microbial biomass

Microbial biomass was determined at the end of the exponential phase of the methylotrophic cultures. A certain volume (40-50 ml) of the culture was centrifuged at 4000 rpm at 15 minutes. The sediment was resuspended in distilled water followed by centrifugation twice. The sediment was then dried at 70°C for 3-5 days to determine the dry weight and protein content. The supernatant of microbial culture was used for formaldehyde determination. The effective yield (Y_E) was also calculated as follows:

$$\text{Effective Yield } (Y_E) = \frac{\text{Microbial biomass mg/L}}{\text{Methanol conc. mg/L}}$$

Chemical determinations of protein and formaldehyde

Protein was determined in the microbial biomass according to Herbert *et al.*^[10] while formaldehyde was determined colorimetrically, using chromotropic acid reagent, at 570 nm^[11].

Electron microscopy examination

The bacterial cells were dried and coated by carbon and finally by gold and palladium^[12]. The cells were examined with Transmission Electron Microscopy.

Identification of bacterial and yeast isolates

The most efficient bacterial and yeast isolates were identified according to Bergey's Manual of Determinative Bacteriology^[13] and Barnett *et al.*^[14] respectively.

Results and Discussion

Methylophilic Isolates

Data presented in Fig. (1) show the number of different microbial isolates obtained from soil samples. Results indicated that 277, 43, 12 and 53 isolates of bacteria, actinomycetes, yeasts and fungi respectively were obtained from 27 soil samples. The bacterial isolates represented the most dominant isolates among all tested soil samples

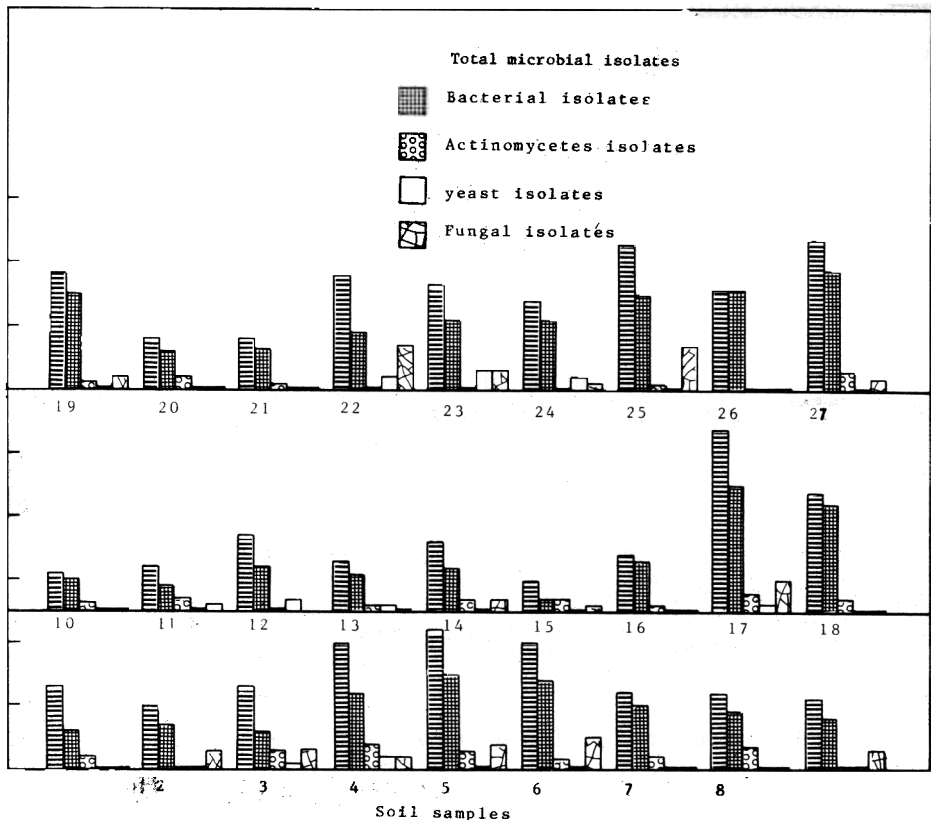


FIG. 1. Number of microbial cultures isolated from different soil samples.

Results in Table (1) also indicated that 26 bacterial isolates out of 277 (9.39%) were found to be methylophilic. The bacterial isolates obtained from 11 soil samples (number 2, 5, 7, 9, 10, 13, 15, 17, 20, 22, and 23) did not grow on methanol mineral medium. The percentage of methylophilic bacteria isolated from other soil samples ranged from 5.26% to 50% of total bacterial isolates. These organisms gave a considerable growth on methanol as a sole source of carbon.

TABLE 1. Methylophilic bacterial cultures isolated from soil samples.

Soil samples	Number of isolates			Methylophilic bacteria
	Total	Bacteria	Methylophilic	
1	13	11	2	18.18
2	10	7	0	00
3	13	6	1	16.67
4	20	12	2	16.67
5	22	15	0	00
6	20	14	3	21.43
7	12	10	0	00
8	12	9	1	11.11
9	11	8	0	00
10	6	5	0	00
11	7	4	2	50.00
12	12	7	1	14.29
13	8	6	0	00
14	11	7	1	14.29
15	5	2	0	00
16	9	8	3	37.50
17	29	20	0	00
18	19	17	1	05.88
19	18	15	1	06.67
20	8	6	0	00
21	8	7	0	28.57
22	18	9	0	00
23	17	11	0	00
24	14	11	2	18.18
25	23	15	2	13.33
26	16	16	1	06.25
27	24	19	1	05.26
Total	385	277	26	100%

With regard to yeasts, 5 isolates were found to be methylophilic. These yeasts were isolated from samples number 4, 17, 22, 23, and 24. Actinomycetes and fungal isolates did not utilize methanol as a sole source of carbon.

Sixteen methylophilic bacterial cultures were obtained from soil samples polluted with petroleum wastes. Other methylophilic were isolated from cultivated soils. Generally it could be stated that 26 and 5 cultures of bacteria and yeasts respectively (out of 385 isolates) were found to be methylophilic. Faust and Prave^[15] reported that marsh land, oil drills, storage tanks, gas transport systems, animal man-

ure deposits, municipal waste fills, natural ponds with boundary zones between aerobic and anaerobic conditions are considered to be the most suitable sources for isolation of methylotrophs. Methylotrophs are quite frequent on organic substances containing methyl groups in ester or ether bonds, such as pectins in fruit pulp, petals of flowers and rotting legnocellulosic materials. Hamdan *et al.*^[16] isolated more than 200 methanol utilizing bacterial cultures from the soil, mud and water near oil fields from different locations in Kuwait.

Growth Rates of Methylotrophs

In this investigation 26 bacterial cultures and 5 yeast cultures were used as methylotrophic microorganisms. The efficiency of these cultures to utilize carbon and derive energy from the metabolism of one-carbon compounds *i.e.* methanol, was observed.

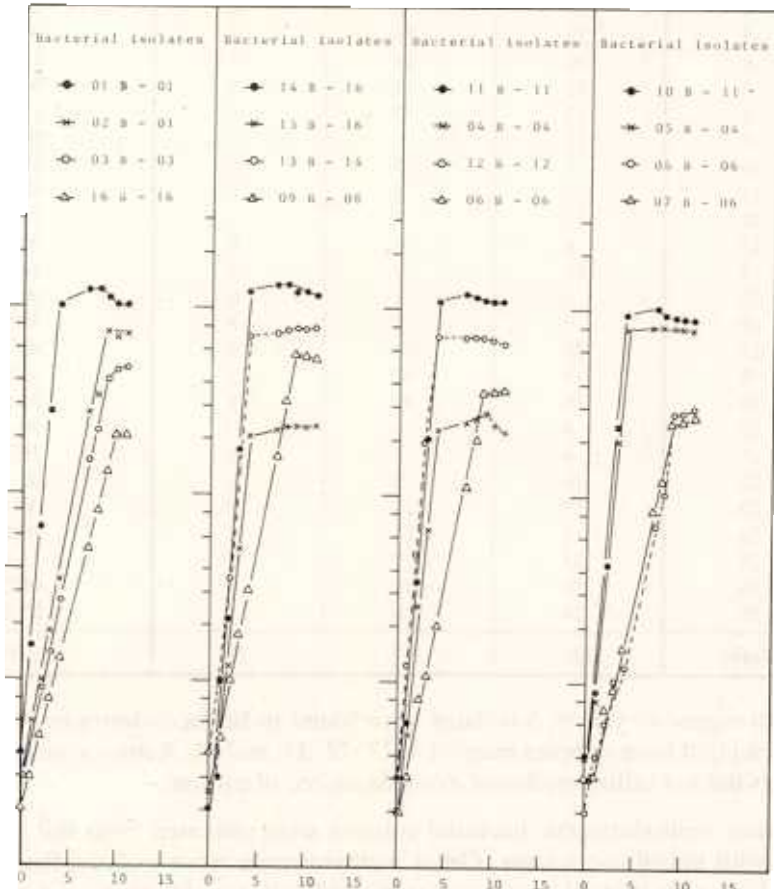
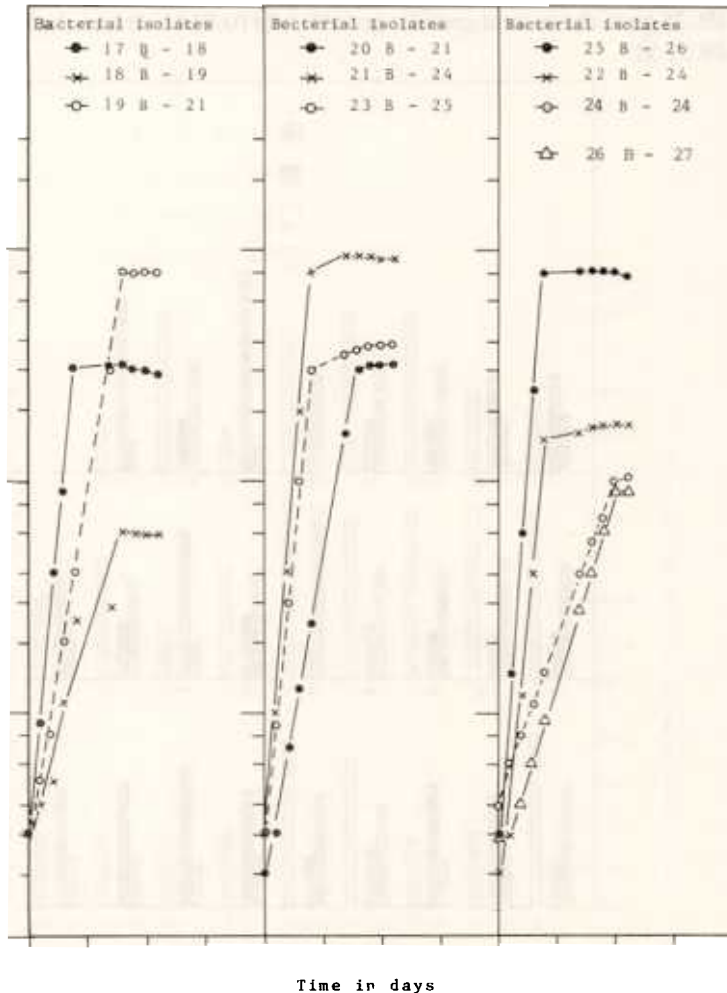


FIG. 2. Growth curves of different methylotrophic bacteria isolated from soil polluted with petroleum wastes.

1. Growth curves of methylotrophic bacteria

The growth curves of 26 cultures of methylotrophic bacteria are presented in Fig. (2 and 3). Results showed that the bacterial isolates 14B-16, 11B-11, 01B-01, 10B-11, 05B-04, 25B-26 and 21B-24 grew exponentially during the first 4 days of incubation without lag phase showing the highest growth at the end of this phase. Their optical density were 12.01, 11.01, 10.41, 9.45, 8.61, 8.07, and 8.02 at 570 nm respectively. Seven cultures also gave their exponential phase during the first 4 days but they showed a low optical density ranged from 1.5 to 7.11. After 4 days of incubation, the growth rate of these cultures decreased gradually (Phase of deaccelerating growth) entering the stationary phase which continued up to the end of experiment (11 days). In contrast, the exponential phase of other bacterial cultures extended up to 8 to 10 days of incubation.



3. Growth curves of different methylotrophic bacteria isolated from cultivated soils.

It could be concluded that the methylotrophic bacterial isolates were represented by 2 groups. The first group showed a clear exponential growth phase, while the second exhibited a very late exponential phase. It was also observed that the methylotrophs 14B-16, 11B-11, 01B-01 and 10B-11 were the most efficient isolates in the utilization of methanol as a sole carbon source.

2. Growth parameters of methylotrophic bacteria

Results indicated that the bacterial culture 14B-16 gave the highest specific growth rate, number of generations and multiplication rate being 0.083, 8.62 and 0.119 h^{-1} respectively (Fig. 4). This was followed by the bacterial cultures 11B-11, 01B-01 and 10B-11 in descending order where their specific growth rates were 0.059, 0.058, 0.057 h^{-1} respectively. These isolates showed the lowest doubling time (8.35-12.16 hours) as compared with other bacterial isolates. On the contrary, the bacterial culture 24B-25 gave the lowest specific growth rate (0.014 h^{-1}) and the highest doubling time (49.51 h).

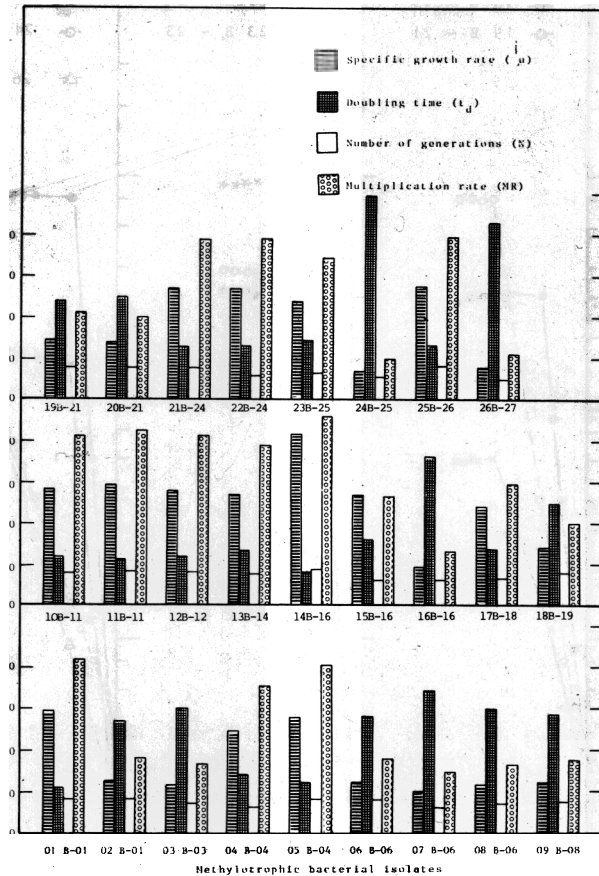


FIG. 4. Growth parameters of different methylotrophic bacterial isolates.

3. Growth curves and growth parameters of methylotrophic yeasts

Five methylotrophic yeasts grew exponentially during the first 3 days of incubation without lag phase (Fig. 5). The yeast isolate 02Y-17 gave the highest optical density after 2 days incubation being 0.62, whereas the lowest figure of (0.13) was recorded in the case of yeast culture 05Y-24.

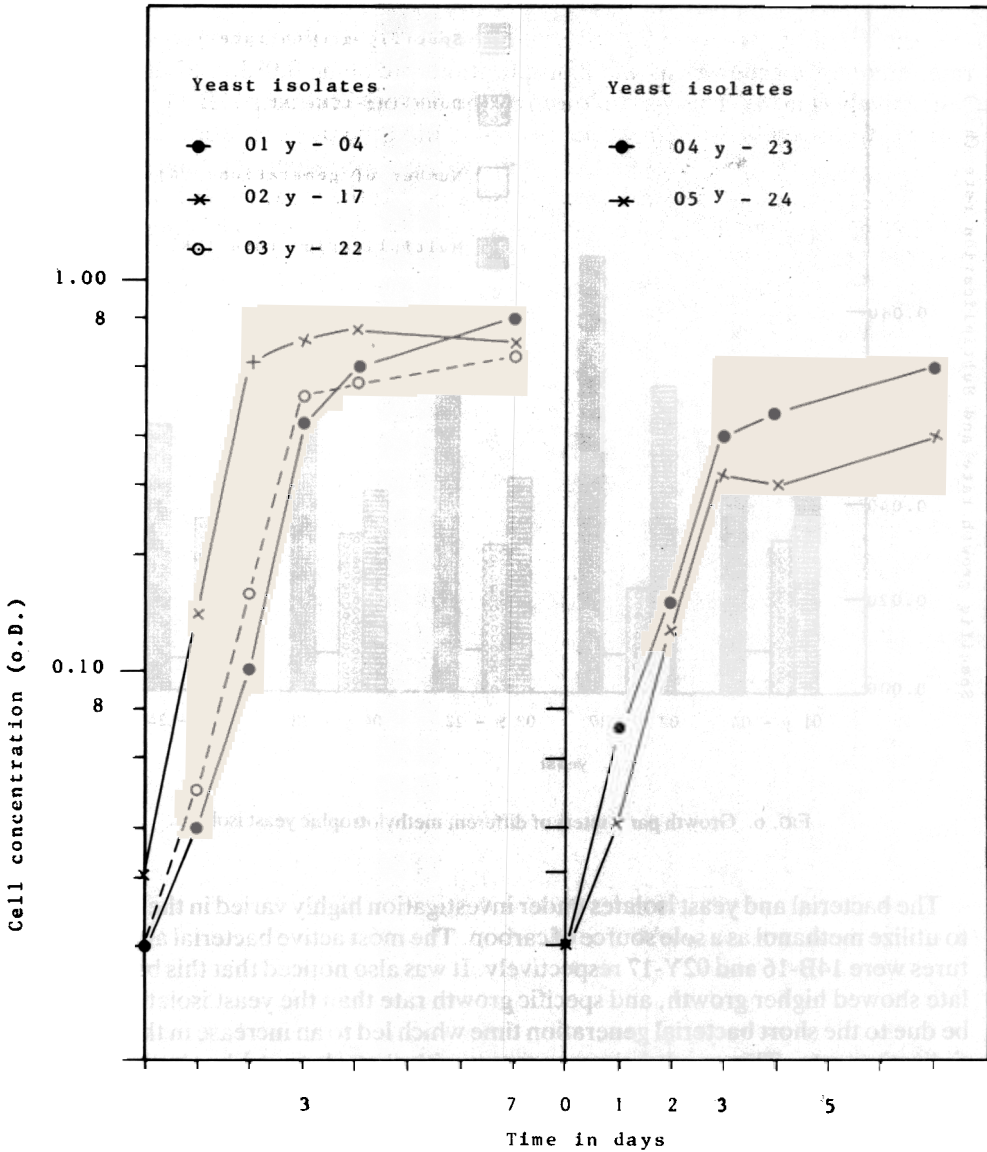


FIG. 5. Growth curves of different methylotrophic yeast isolates.

The isolate 02Y-17 also showed the highest values of specific growth rate, number of generations and multiplication rate (Fig. 6) being 0.063 h^{-1} , 4.36 and 0.091 h^{-1} , respectively. In contrast, the shortest doubling time was observed in the case of this isolates (11 h.).

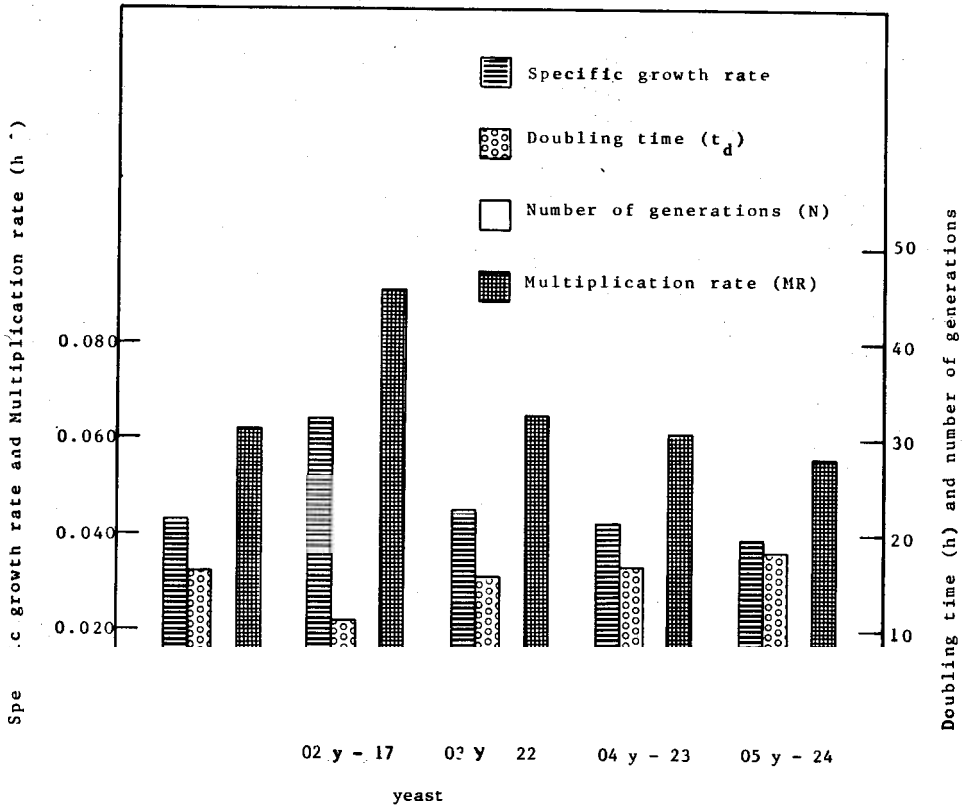


FIG. 6. Growth parameters of different methylotrophic yeast isolates.

The bacterial and yeast isolates under investigation highly varied in their efficiency to utilize methanol as a sole source of carbon. The most active bacterial and yeast cultures were 14B-16 and 02Y-17 respectively. It was also noticed that this bacterial isolate showed higher growth, and specific growth rate than the yeast isolate. This may be due to the short bacterial generation time which led to an increase in the cells multiplication rate. This result is in agreement with that observed by Anthony^[1] who noticed that the critical difference between bacteria and yeasts is in the system for initial oxidation of methanol to formaldehyde. In bacteria, this is coupled to an electron transport chain, and thus to ATP synthesis, but this is not the case in yeast where a flavoprotein oxidase is responsible for oxidation of methanol.

Identification of the Most Efficient Methylophilic

Methylophilic bacterial isolates 01B-01, 11B-11, 14B-16 and 10B-11 were gram-negative short rods, motile by polar flagellum (Fig. 7). These isolates are characterized by the formation of pink cells reflecting the presence of carotenoid pigments. They utilize methanol and can not grow on nutrient agar. These isolates are considered to be obligate methylophilic and strict aerobes. They utilize ammonium sulfate and nitrate as a sole nitrogen source. According to Bergey's Manual of Determinative Bacteriology^[13], *Methylophilus* is the proposed genus and *M. methanica* is the type species of these four isolates. Due to the difference between these isolates in their efficiency to utilize methanol, they may be considered as strains of *M. methanica*.

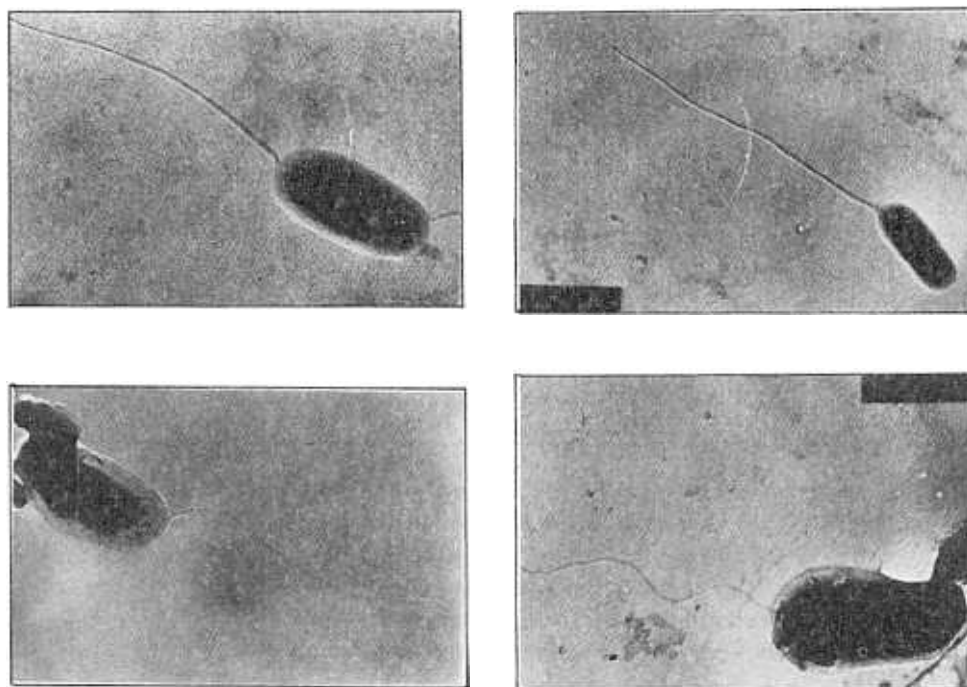


FIG. 7. Electron micrograph of four strains of *Methylophilus methanica*, showing single polar flagellum (The dried cells were coated with carbon and finally fixed by gold and palladium).

With regard to yeast isolates, they are characterized by the formation of multilateral budding cells, round ascospores. *Pichia* is the proposed genus for these isolates according to Barnett *et al.*^[14]. These yeasts are also considered to be facultative methylophilic.

Biomass the Protein Productivity

Table (2) shows that *Methylomonas methanica* 14B-16 gave the highest biomass being 113.30 mg dried cells/100 ml culture after 4 days incubation. Protein percentage and protein productivity (mg protein/100 ml culture) exhibited the same trend being 65.2% and 73.88 respectively. The lowest values were observed in the case of *Pichia* sp. 02Y-17. It was also noticed that the lowest biomass was detected only in the cultures containing high concentration of formaldehyde (*M. methanica* 10B-11 and *Pichia* sp. 02Y-17). This may be attributed to the higher oxidation rate of methanol to formaldehyde than the fixation rate of formaldehyde with pentose phosphate. On the other hand the accumulation of formaldehyde in the cultures led to decreasing the biomass due to the toxicity of this compound at low concentrations. Pilat and Prokap^[17] reported that formaldehyde is more toxic than methanol. Papoutsakis et al.^[18] also added that the inhibition caused by formaldehyde occurs at 0.022 mM while at 0.223 mM, the inhibition is complete. In our investigation, the maximum yield of formaldehyde was 621 $\mu\text{g}/100\text{ ml}$ (0.21 mM).

TABLE 2. Biomass of methylotrophic microorganisms and their protein productivity.

Microorganisms	Growth mg/100 ml	$\mu\text{ h}^{-1}$	Effective yield (Y_E)	Formaldehyde $\mu\text{g}/100\text{ ml}$	Protein %	Protein Productivity mg/100 ml cult.
<i>Methylomonas methanica</i> Strains						
01B-01	99.06	0.058	31.34	55	62.1	61.51
011B-11	103.86	0.059	32.87	51	61.3	63.67
14B-16	113.30	0.083	35.85	40	65.2	73.88
10B-11	89.15	0.057	28.21	424	60.4	53.85
<i>Pichia</i> sp. 02Y-17	50.02	0.063	15.83	621	48.2	24.11

The amount of biomass per unit of methanol in the medium (Effective yield) was high (35.85%) in the case of *M. methanica* 14B-16 as compared with other bacterial and yeast strains. It means that this strain has a high efficiency to utilize methanol than others. This is in line with that observation made by Anthony^[1]. On the other hand, single-cell protein derived from industrial-scale process based on methanol have been declared safe and applicable for animal nutrition^[15].

Finally, it could be concluded that bacteria have a clear advantage over yeast in their protein content, growth rate and effective yield. The use of methanol assimilating bacteria for production of single-cell protein may be participated in the solving of protein shortage specially in the countries producing methanol as a high pure substrate and low cost relative to other carbon sources such as Kingdom of Saudi Arabia.

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اختيار الكائنات المثلية التغد لتكوين وتيات الخلية الوا

كمال بن عباس بويين فهد الرحمن الفاسي
قسم علوم حياء كذله العلوم الملك العزيز
المملكة العربية السعود

عزلة عزل الكائنات الحية الدقيقة المثلة للميثانول من
مثلية التغدة بكتريا خائز، من ولم يظهر كل
والاكثويو— أي تد على الميثانول وحد للكربون
اختلفت معايير النمو لعزلات البكتريـ الخمير اختلافاً واضحاً من
لمراوح اليهتسز وأظهـ البكتريـ الميثيقيه التغذية (١٤)ب
ب١ ب١ أعلى خصصي
ساعة) على التسوالي وقد أعطيت البكتريا
فترة نمو اسبي قصيرة مقارنة بالبكتريـ وكانت اعلى كتلة حيويه وإنتاجه
للبروتين (٣) مجم سم مزجعة) لسلالة البكتريا الميثيلية التغديه
معامل النمو منحصاً بالتركيزات العائبة للثور.