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Isolation and Characterization of Two Thermotolerant Methylotrophic Bacterial Strains

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عزل وتعيين خصائص بعض سلالات البكتيريا المحُبّة للحرارة العالية والمؤكسدة لمادة الميثانول

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أجريت عمليات عزل وتعيين خواص عدد من السلالات البكتيرية المحبة للحرارة العالية والمكونة للأبواغ من عينات تربة سطحية أظهرت الدراسة أن أحد هذه الأصناف والتي أطلق عليها إسم EM1 تنتمي إلى جنس العصويات وأخرى EM2 تنتمي إلى جنس الاكتينوميستات.

تمت اختبارات الكشف عن قدرة هذه العزلات على النمو في البيئات التي تتركب من بعض الأملاح المعدنية مذابة في الماء مع إضافة 50 ملي مول من مادة الميثانول كمصدر وحيد للكربون والطاقة. وقد تمت أيضاً دراسة بعض العوامل المؤثرة على النمو مثل تحديد درجة الحرارة المثلى لنمو البكتيريا وتأثير إضافة تراكيز مختلفة من مادة الميثانول وتحديد الرقم الهيدروجيني pH المثالي لنمو نفس البكتيريا.

وجد أن درجة الحرارة المثلى لنمو السلالتين تتراوح ما بين 50 إلى 55 درجة مئوية وباستخدام تقنيات المزرعة المستمرة تمت دراسة بعض العوامل المؤثرة الأخرى مثل استهلاك الميثانول وغاز الأكسجين وانتاج غاز ثاني أكسيد الكربون وحساب معدل النمو ونواتج عمليات الأيض الحيوي عن طريق الوزن الجاف للخلية والمحتوى البروتيني داخل الخلية.

Abstract *Two thermotolerant methylotrophic bacterial isolates* (EM_1, EM_2) *capable of growing in mineral salts medium, containing 50 mM methanol as the sole carbon and energy source, were studied. The maximum specific growth rates of* EM_1 *and* EM_2 *in batch culture were* $1.39\,h^{-1}$ *and* $0.8\,h^{-1}$ *respectively.*

Growth of the two tested microorganisms was influenced by different (growth conditions) methanol concentration, temperature and pH *values. They differed in their tolerance to methanol concentration. The optimum growth temperature of* EM_1 *and* EM_2 *were 50 and 55°C respectively. The highest specific growth rate* (μ_{max}) *of* EM_1 *and* EM_2 *were observed at the optimum* pH *7.4 and 8.0 respectively.*

In chemostat continuous culture of the isolate EM_1 *there was an increment in the different growth parameters: methanol concentration, oxygen consumption and* CO_2 *production. Moreover the metabolic and cell growth rate monitored as cell dry weight (mg/ml) and protein content in whole cells were also increased.*

INTRODUCTION

Methylotrophic bacteria play important roles in several biotechnological processes (Anthony 1982; Crawford and Hanson 1984; van Verseveld and Duine 1987). They are most interesting organisms particularly from an applied point of view since evidence has been published for the presence of methylotrophic bacterial strains in nature (Colby and Zatman 1975; Akiba *et al.*, 1970; Snedecor

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and Cooney 1974; Hitzman 1976), Consequently, these menthanol – utilizing thermotolerant bacterial strains may be of considerable industrial interest, both as single cell protein production (s.c.p.) and also for the transformation of methanol to useful metabolites. They have the ability to grow easily on this inexpensive carbon source. The use of microorganisms, capable of growing on petroleum derived fractions, such as methane and methanol, has been established.

The balance of mass – energy must be examined, thus, Yoshifumi *et al.*, (1983) found that there was an exponential increase of bacterial growth lag with the increase in methanol concentration.

Other growth conditions and regulation of methylotrophic bacteria were previously reported. (Dijkhuizen *et al.*, 1988). Methylotrophic bacteria are varied in their physiological behavior with respect to different growth conditions. Methanol utilizing bacteria are also classified according to their efficiency optima. The most important biotechnological factors for s.c.p. production are cell yield, growth rate, and crude protein content in whole cells of biomass (Lowry *et al.*, 1951).

Other parameters for biotechnological factors are substrate affinity, thermotolerance and the biological stability of the culture.

The objective of this work is to study two isolated local thermotolerant methylotropic bacteria and the effect of some environmental conditions on their growth.

MATERIALS AND METHODS

The bacterial strains were isolated on aerobic medium of the following composition (per litre of distilled water): $(NH_4)_2\ SO_4$ 1.5 g, K_2HPO, 4.65 g, $Na_2HPO_4.2H_2O$ 1.5 g $Mg\ SO_4.7H_2O$ 0.2 g, 2ml/litre of trace elements solution (Vishniac and Santer 1957) and 1.5% w/v of agar for isolates maintenance, was added to the same medium.

The medium was sterilized in autoclave at 121°C for 15 minutes. The microorganisms used throughout this study have recently been isolated in pure culture in our laboratory from soil samples, where one gram of each sample was introduced into 150 ml conical flask (in triplicates) containing 25 ml of the above medium. Appropriate amounts of (filter sterilized) methanol were added to each flask. The inoculated flasks were incubated in a horizontal shaker (80 rpm) at 55°C for three days. Culture suspensions of 72 h incubation were streaked out on methanol agar plates and incubated at 55°C for 48 h. Two types of colonies, designated as EM_1 and EM_2, producing good growth on methanol agar, were selected for subsequent experiments.

GROWTH EXPERIMENTS

Growth of the isolated bacterial strains on different methanol concentrations (25, 50, 100, 200, 300, 400, 500, 600 and 800 m mol) were examined in liquid medium. The effects of temperature and pH values on the growth of the isolated strains were carried out. Growth was monitored by measuring the optical density at 430 nm. Doubling time (Td) and the maximum specific growth rate (μ_{max}) of the two isolates (EM_1 and EM_2) were calculated according to the following equations:

$$Td = \frac{t_2 - t_1}{ldx(t_2) - ldx(t_1)} \quad (1)$$

$$\mu_{max} = \frac{In2}{Td} = \frac{0.693}{Td} \quad (2)$$

Continuous Culture Cultivation

The growth of strain EM_1, in continuous culture at 50°C and pH 7.4 was used to study the effect of various dilution rates. During continuous growth, methanol and oxygen consumption rates and CO_2 production were estimated as described by Brooke *et al.*, (1989). Dry weight was measured by gravimetry after drying overnight at 105°C, as previously conducted by Dijkhuizen *et al.*, (1988). Protein content in whole cells was estimated previously as stated by Duchards and Attwood, (1989) and Brooke *et al.*, (1989). Bovine serum albumin was used as standard protein.

RESULTS

The two isolated EM_1 and EM_2 were found to produce a good growth on agar medium containing 50 mM methanol, however these strains differed in several characteristics. EM_1 was gram positive, rod shaped cells of length up to 8.57–11.43 μm and spore forming bacilli. The other strain EM_2 was also gram positive but the cells showed filamentous shape non spore forming bacteria. Both strains do not utilize glucose as a carbon source and do not grow on nitrate, asparagine and methylamine HCI but they

can easily grow on glutamine as a nitrogen source other than ammonia.

Effect of Methanol Concentration on the Specific Growth Rate

The specific growth rate of the two tested bacterial strains was significantly affected by the high methanol concentration (Fig. 1). The highest specific growth rate (μ_{max}) 1.39 h^{-1} of the strain EM_1 was observed on methanol concentration range 0.1–0.2 mol. The specific growth rate (μ_{max}) suddenly decreased when the methanol concentration was more than 0.2 mol. The response of the strain EM_2 was different. This strain showed high sensitivity to the low methanol concentration. The specific growth rate (μ_{max}) of this strain was in the range of 0.8–0.84 h^{-1} in methanol concentration of up to 0.1–0.2 mol.

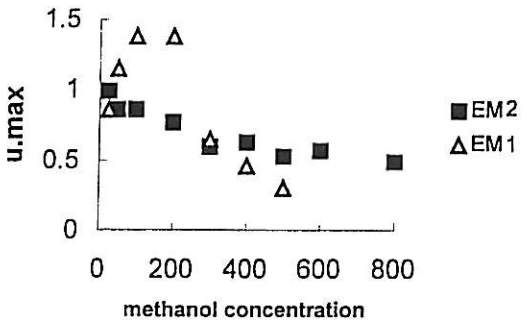

Fig. 1. Influence of different methanol concentration on the growth of two tested bacterial strains.

Effect of Temperature

The two bacterial strains were able to grow at a wide range of temperature 37–60°C (Fig. 2). The highest (μ_{max}) of the strain EM_1 1.3 h^{-1} was observed at 50°C and this value slowly decreased due to the increase of the growth temperature, however the highest (μ_{max}) value 1.0 h^{-1} of EM_2 was observed at 55°C.

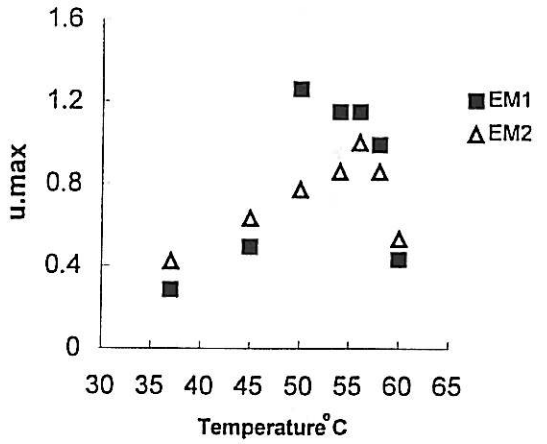

Fig. 2. Influence of the cultivation temperature on the growth of both strains.

Effect of pH

The results showed that the (μ_{max}) of the two bacterial strains were influenced by pH value (Fig. 3). The optimum pH values for the growth of EM_1 and EM_2 were 7.4, 8.0 respectively.

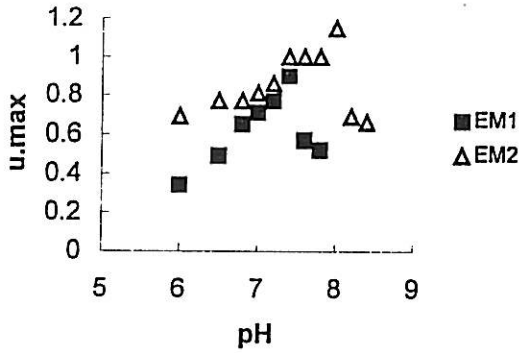

Fig. 3. Typical (μ_{max}) of different pH spectrum.

Effect of Dilution Rate on the Growth of the Bacillus Strain EM_1

As the above results revealed that the strain EM_1 was efficient to grow on methanol under optimal conditions, this strain was chosen for further investigations of the effect of dilution rate on the different parameters of continuous growth in chemostat culture. The results indicated that the increase of methanol dilution rate (D. rate) is associated

Table 1. Influence of growth rate on the kinetics of growth of bacillus strain EM1 in a carbon–limited chemostat culture (temp = 50.0°C, pH = 7.4, SR = 50.0 mM methanol.) Rates: [mM/h^{-1} (g dry weight/cells)$^{-1}$].

D. rate (h^{-1})	*q methanol*	*qo2*	*qco2*	*yield g/mol*	*dry wt mg/ml*	*protein %*
0.11	7.4	5.15	2.7	14.8	0.74	45.9
0.155	9.6	6.75	2.9	16.2	0.81	45.68
0.21	13.0	8.67	3.43	17.6	0.88	55.68
0.25	14.6	9.8	3.5	18.5	0.925	56.2
0.32	16.9	11.51	3.6	19.0	0.95	58.9

SR = concentration of methanol in the reservoir.
D = dilution.
q = consumption and/or production.

with an increase in the methanol and oxygen consumptión and CO_2 production. Influence of dilution rate on growth of the thermotolerant methylotrophic bacilli EM_1 in continuous culture at optimum temp 50°C, pH 7.4 and methanol concentration 50 mM is shown in Table 1. As the cultivation D. rate was increased the bacterial dry weight increased from 0.74–0.95 mg/ml at D. rate 0.11 h^{-1} to the maximum of 0.32 h^{-1}. The data indicated that the increase in protein content in whole cells is correlated with the D. rate and methanol consumption.

DISCUSSION

Microbial biomass production can be represented by different microbial growth parameters out of these is the maximum specific growth rate μ_{max}. From an economic point of view, the efficiency of an industrial growth process is increased with higher μ_{max} and in mixed culture the species with the greatest (μ_{max}) has the highest probability of survival Guthkre (1980). The results of this work revealed that μ_{max} of the tested bacterial strains EM_1 and EM_2 reached their highest values when the medium contained methanol in concentration range 50–200 mM. The influence of the methanol concentration on methanol utilizing bacteria growth and activity was not fully established. Girio and Attwood, (1991) found that the addition of 50 mM methanol as a carbon limited chemostat culture of methylobacterium species caused an acceleration of its metabolic rates. There is a relationship between methanol concentration and μ_{max} of Methylotrophic bacteria. Yoshifumi Amano *et al.*, (1983) found that the highest maximum specific growth rate was determined at methanol concentration lower than inhibition constant (ki) 40 g/l. The date indicated that the μ_{max} of the two bacterial strains was influenced by the growth temp. The results showed that the two isolates have the highest μ_{max} at temperature range 50–55°C which could strongly suggest that the two bacterial strains are methylotrophs thermotolerant bacteria. The thermophilic bacteria are classified with respect to pH into two major groups (Sonnleiter, 1983; Sundaram, 1986).

- Thermoacidophiles with pH optima in the range 1.5-4.0.
- Thermophilic neutrophiles with pH optima in the range 5.8-8.0.

Our bacterial strains have the highest μ_{max} value at the pH range 7.4–8.0. Thereby it could be suggested that these strains EM_1 and EM_2 are methylotrophic thermotolerant neutrophilic bacteria.

The resulting increase in the cell dry weight and protein content during continuous cultivation of the Bacillus efficient strain EM_1, the increase in methanol and oxygen consumption and the increase in D. rate, show a linear relationship.

Maintenance Energy Requirement

When q methanol is plotted against D. rate the resultant plot yield information regarding the maximum growth yield ($Y^{max}_{methanol}$) on methanol and maintenance energy requirement. Linear regression analysis of the date gave a $Y^{max}_{methanol}$ of 35.7 g cells. mol methanol.

The increment in cell dry weight and protein concentration suggested that there is polymer storage produced throughout the D. rate increment (Kjeldgaard and Kurland, 1963), whereas synthesis of their catabolic enzymes is not similarly adjusted (O'Brien *et al.*, 1980).

These anabolic, enzyme activities, and identification of some metabolic products, need further investigations.

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