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Research Note

Feed Stuff Production from Methanol by Methylotrophic Microorganisms

P. Jafari[1,2], G.M. Zamani[1,*], F. Almasian[1], B. Medi[1], N. Ghaemi[1], A. Nohi[1] and S. Mehrabian[1]

Methanol has been of great interest as a substrate for production as a novel feed grade protein in Iran. Up to 160 methylotrophic strains were isolated at the Jahad-e Agriculture Research Engineering Center for Scientific Research and various batch experiments were carried out at different pH, temperature, agitation, aeration rate and methanol concentrations. By these experiments, optimum production conditions were determined for one of the strains using the Taguchi method. The results showed optimum pH around 8 at temperatures of 26 to 30°C, aeration equal to 3 vvm and agitation about 800 rpm. The methanol was found to be optimum at concentration equal to 18 $g.l^{-1}$. In this condition, the biomass yield coefficient was 0.52 g/g methanol, biomass dry weight was 20-25 g/l and the crude protein content was about 70-78%. A deep jet fermentor of 1 m^3 capacity, with a working volume of 600 liters, was used for SCP production using isolated strains. In this continuous system, dilution rates ranging from 0.15 to 0.2 $lit.h^{-1}$ were used to establish optimal condition for biomass production. Under this condition, 30 kg cell dry weight per day was obtained. These experiments showed that the selected methylotrophic strain has good potential for SCP production from methanol as a substrate.

INTRODUCTION

World resources for food production by conventional methods are limited in being able to satisfy the needs of an increasing global population. If the world population continues to rise at its present rate, the demand for animal protein will increase considerably, as will the need for feed, cereals and see oil meal, since the present trend in crop production cannot fill this rising gap [1]. Nowadays, in many countries, huge amounts of Single Cell Protein (SCP) from petroleum feedstock is already been produced for animal feed. For example, Norferm AS, a joint venture between DuPont and Statoil, has developed and commercialized a unique continuous fermentation technology for the production of biomass based on natural gas. The product, BioProtein autolysate, derived from this biomass, is a complex N-source with a proven performance in enhancing the economic performance of different fermentation productions.

SCP as a feedstuff is completely safe [2]. In view of this potential, the Persian Gulf states, with abundant petroleum feedstuff and energy resources, hold great promise for the protein needs of the developing world. So, the conversion of methanol to more valuable products, such as SCP, is an appropriate strategy for overcoming the shortage of protein in these countries [3,4].

A great number of bacteria, called methylotrophic microorganisms, consume methanol as both carbon and energy sources and produce protein. Because of the fast growth rate and high protein content, the culture and use of this type of microorganism could be suitable for SCP production [5].

The rising demand of protein ingredients in Iranian animal husbandry has increased research in this field. An integrated single cell protein and development program, using methanol as a substrate, was initiated at Jahad-e Agriculture Research Engineering Center. The research and development activities of this program comprise:

1. *Biotechnology Division, Jahad-e Agriculture Engineering Research Center, P.O. Box 13445-754, Tehran, Iran.*
2. *Sciences Research Branch, Azad Islamic University, P.O. Box 14515-775, Tehran, Iran.*
*. *To whom correspondence should be addressed. E-mail: gmzamani@yahoo.com*

a) Technoeconomic feasibility study of process routes with potential application in Iran,

b) Isolation and physiological studies of microorganisms from the Iranian environment, selection of the most suitable process for local conditions,

c) establishment of an SCP pilot plant facility to produce the SCP product.

This pilot plant will enable one to conduct tests, render a pilot production of feed protein and, finally, to translate the findings gained from this process into a planned large scale production plant. This pilot plant can be used to grow the inoculums for a large scale plant of 30,000 tpy SCP planned capacity.

For the economic commercial scale production of SCP from methanol, continuous culture systems are more attractive than batch culture systems, because a higher yield is attainable in continuous systems. However, irrespective of which two culture systems are involved, it is crystal clear that effective variables must be optimized to as great an extent as possible [6,7]. This usually includes studying growth conditions, metabolism and kinetics in a laboratory, with a shaking flask scale. In this study, the necessary data required to design commercial scale production is provided [8,9].

MATERIALS AND METHODS

Isolation and Growth Media

A simple mineral medium, named methanol salt, was used for bacterial isolation and growth. It contained methanol (5 g/l) and KNO_3 (3 g/l) as carbon and nitrogen sources, respectively. The phosphorus source was phosphate salts or phosphoric acid. Salts were used to supply the strains with potassium and magnesium and trace elements, such as copper, cobalt, manganese, zinc and molybdenum. Boron was added as boric acid. Analytical grade chemicals were used in the experiments and the salts and other reagents used for the cultivation medium preparation were from Merck, Germany. A solid medium was prepared by adding 2% (w/v) purified agar. The medium was adjusted to pH 7 ± 0.2 with 0.1 M NaOH. The medium was autoclaved at 121°C for 15 minutes and then filter sterile methanol was added.

Microorganisms

C1-assimilating bacteria were isolated from water and soil samples taken from the Ahwaz oil field. The samples were added to a 250 ml Erlenmeyer flask containing 50 ml of methanol salt medium and incubated for 2 weeks at 30°C on a rotary shaker. Up to 160 different strains were isolated from this methylotrophic enrichment culture. The isolates were maintained, both on methanol salt agar medium slants and in a 50% glycerol medium. All of these strains were grown in a methanol salt medium and 2 of them were selected because of their higher yield.

Morphological and Biochemical Observations

Gram stain reaction and lysis at 3% (w/v) KOH were carried out with both 2 and 4 week old cultures. Poly-β-hydroxybutyrate inclusions were determined by staining with 0.03% (w/v) sudan black and 0.5% safranin. Observation of rosette formation, as well as checks for the presence of exospores or cysyts, was carried out during all phases of growth on both liquid and agar media. Colony morphology and pigmentation were examined with 2, 4 and 6 week old methanol salt agar plate cultures. Biochemical characteristics, such as motility, oxidase, catalse and etc. were determined for isolated strains.

Fermentation Technique

A laboratory scale stainless steel stirred fermentor with a 2 liter capacity and a working volume of 1 liter was used for SCP production. Different cultural conditions, such as inoculum size (from 5 to 25%), methanol concentration (from 3 to 22 g/l), incubation temperature (from 20 to 40°C), initial pH (from 5 to 8), air supply (from 1 to 4 vvm) and agitation rate (from 100 to 1000 rpm) were optimized for enhanced SCP production. In these experiments, all variables and conditions, except one, were constant. Via these experiments, the effects of changes in culture variables and conditions on SCP production were determined. For this purpose, samples were taken from the sterile port of a fermentor every 2 to 3 hours. The OD of each sample was measured by using a spectrophotometer.

Design of Taguchi Experiments

The locally optimized points obtained from the above experiments were used to design the optimization experiments using the Taguchi approach. An L-9 array (4 three level factors) was used to design this experiment (see Tables 1 and 2) and all assays were conducted in triplicate. The analysis of results was done by means of Qualitek-4 software.

Analytical Method

Optical Density

Turbidometry, as the most convenient, simple, and widely used method of estimating the total biological

Table 1. Design of L-9 array for Taguchi approach.

Level / Parameter	Level 1	Level 2	Level 3
Methanol (g/l)	15	12	18
Aeration (vvm)	3	2.5	2
Agitation (rpm)	800	600	400
pH	7	6	8

Table 2. Taguchi trials for experiments optimization.

Trail	Methanol	Aeration	Agitation	pH
1	15	3	800	7
2	15	2.5	600	6
3	15	2	400	8
4	12	3	600	8
5	12	2.5	400	7
6	12	2	800	6
7	18	3	400	6
8	18	2.5	800	8
9	18	2	600	7

material in suspension, was selected for growth analysis. The optical density of the biological content of each Erlenmeyer flask was measured at 600 nm.

Cell Dry Weight

For determination of bacterial cell weight, 50 ml of bacterial culture was centrifuged at 5000 rpm for 10 minutes. The pellet was dried at 110°C overnight and the dry weight was determined.

RESULTS AND DISCUSSION

Morphological and Biochemical Test

Selected strains were gram-negative and short rod shaped. These strains produced pink-red growth pigment. Such strictly aerobic bacteria are called pink pigmented facultative methylotrophic microorganisms. Their colony, on the methanol salt agar, was circular, with a 3-5 mm diameter. Biochemical and molecular identification showed that two selected strains belong to a genus of methylobaterium, the cultural and biochemical characteristics of which are shown in Table 3.

Production Optimization in 2 Liter Fermentor

Effect of Initial Methanol Concentration

Methanol concentration was one of the most effective factors for the growth of the selected strains. The optical densities of both strains in the medium with different initial methanol concentration (g/l) are shown in Figure 1. The optical densities rise monotonically until optimum concentration and then decline slowly at higher initial methanol concentrations for both strains. This optimum value is about 18 g/l for both strains. In the optimization experiment, methanol concentration in the range of 10 to 12 g/l was used. As shown in Figure 1, in this range of methanol concentration, the highest amount of biomass was gained. With a methanol concentration below this range there was no sufficient carbon source supporting bacterial growth and in a methanol concentration in excess of this range, the toxic effect of the methanol inhibited bacterial growth.

Effects of Agitation (rpm) on Growth

The results indicated that an agitation rate of about 600 rpm was the best for growing both strains. As is obvious in Figure 2, biomass production in the range

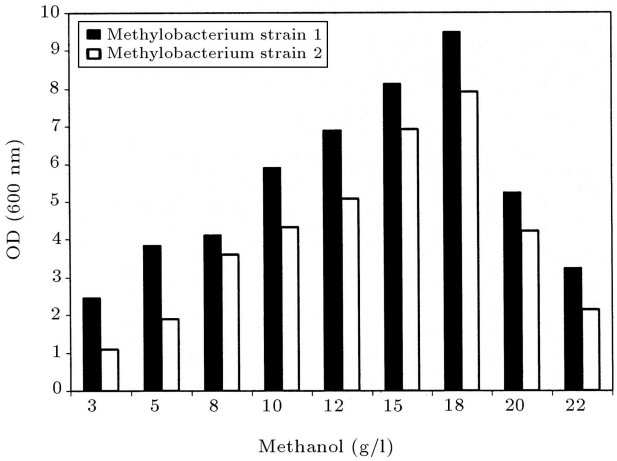

Figure 1. The variation of optical density of Methylobacterium strains 1 and 2 versus initial medium methanol concentration.

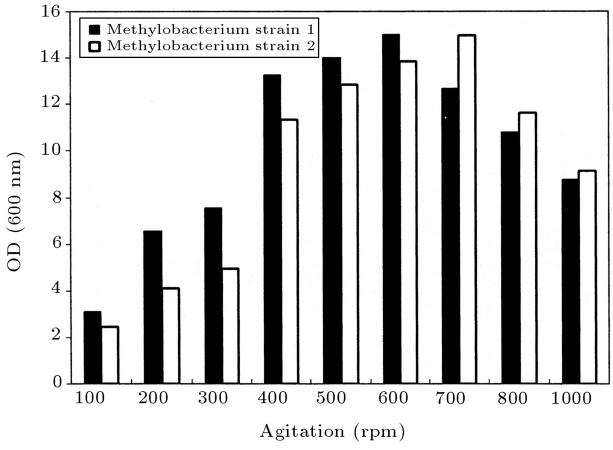

Figure 2. The variation of optical density of Methylobacterium strains 1 and 2 versus agitation.

Table 3. Cultural and biochemical characteristics of Methylobacterium.

Characteristics	Methylobacterium
Gram reaction	− or v
Cell shape	
Rod or Coccobacilli, straight or curved	+
Cocci	-
Cell diameter, μm	0.8-1.0
Cyst or cyst like form occur	
Cell arranged in square tablets of 16-64 cells	−
Motility in liquid media	+
Flagellar arrangement	
Polar or sub polar	+ or −
Lateral	+ or −
Poly hydroxybutyrate accumulated	+
Growth at 60°C or higher	−
Growth at 5°C not at 37°C	
Florescent pigment	
Yellow colonies	−
Red or orange colonies	+
Violet colonies	−
Oxidase	+ or W
Acid from glucose	
Indole from tryptophan	
Oxidize ethanol to acetic acid at pH 4.5	−
Fix N_2 in vitro aerobically	
Fix N_2 in vitro under microaerobic condition	
Fix N_2 in root nodule of plant	−
Denitrifiaction (to N_2)	
Growth with 20% or more NaCl	−
Facultative H_2 autotrophs	−
Genus include pathogens	
Human and/or animal	−
Plants	−

of 400 to 800 rpm did not show any significant change, but in agitation rates above and below this range, the amount of produced biomass decreased.

Effect of Temperature

The optical density of the selected strains at different incubation temperatures, from 20 to 40°C, is shown in Figure 3. It is clear that both strains are mesophile and their optimum growth temperature is around 30°C. The highest optical densities were seen from 26°C to 30°C. Bacterial growth at higher temperatures is a batch and a continuous system, so 30°C was selected as the bacterial growth in all other experiments.

Effect of Initial pH on Growth

The optimum value of initial pH is about 7, as can be seen from Figure 4, in which the variation of optical density by changes in initial pH is shown. The initial pH values from 6 to 8, regarding their highest corresponding optical densities, were selected for the optimization experiments.

Effect of Air Supply on Growth

The optical density of selected strains with different air supplies during the fermentation process is shown in Figure 5. Maximum optical density under this condition was obtained in the range of 2 to 3 vvm.

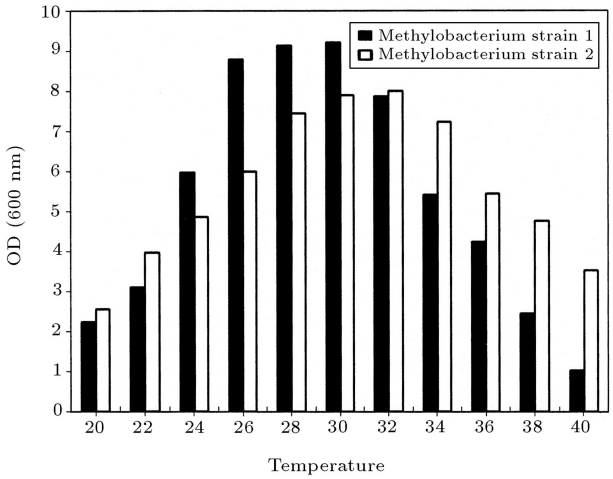

Figure 3. The variation of optical density of Methylobacterium strains 1 and 2 versus incubation temperature.

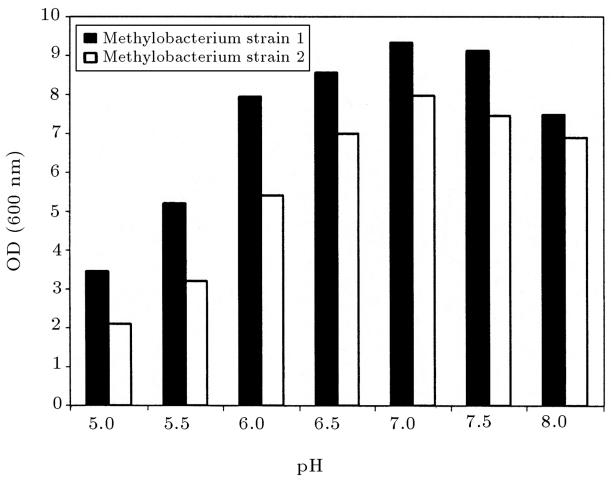

Figure 4. The variation of optical density of Methylobacterium strains 1 and 2 versus initial pH.

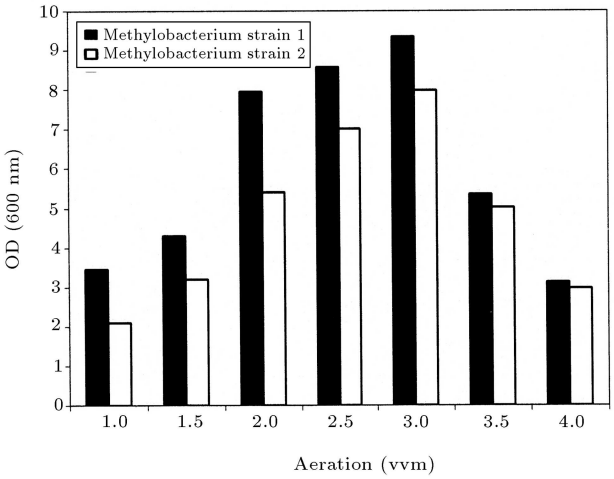

Figure 5. The variation of optical density of Methylobacterium strains 1 and 2 versus aeration.

So, in the Taguchi experimental approach, this range was applied.

Growth Kinetics of Selected Stains

In this experiment, the growth kinetics of Methylobacterium strains were determined in a 1 liter fermentor. The experiment was carried out under optimum conditions. Agitation speed was set to 600 rpm, while temperature, pH and methanol concentration were 30°C, 7 and 18 g/l, respectively. The growth kinetics of the selected stains are shown in Figure 6. Under these conditions, the maximum optical density obtained in the batch culture with no pH control, were about 9 and 8 for Methylobacterium strains 1 and 2, respectively.

Taguchi Optimization

The results of the designed experiments for SCP production optimization by Methylobacterium strain1 is shown in Figure 6 (Trail 1 to 9). These data were analyzed by Qualitek-4 and the optimized growth condition for this strain was extracted. Via these experiments, optimum production conditions were determined for one of the strains, using the Taguchi method. The results showed an optimum pH of around 8, at temperatures of 30°C, agitation about 800 rpm and an aeration volume of about 3 vvm. Methanol was found to be optimum at a concentration equal to 18 g l^{-1}. Another experiment was carried out under these conditions and the results are shown in Figure 7 (Trail 10). It was obvious that the amount of SCP production under these new conditions was much higher than in the previous experiments.

The overall reaction occurring when the Methylobacterium strain 1 was grown on methanol can be summarized by the following approximated stoichiometry:

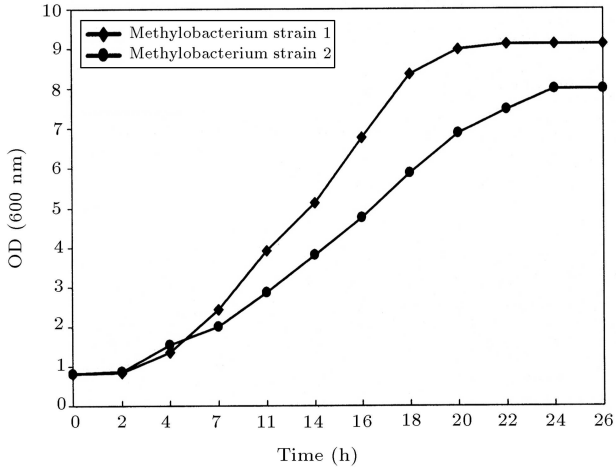

Figure 6. Growth kinetic Methylobacterium strains 1 and 2 in optimum conditions.

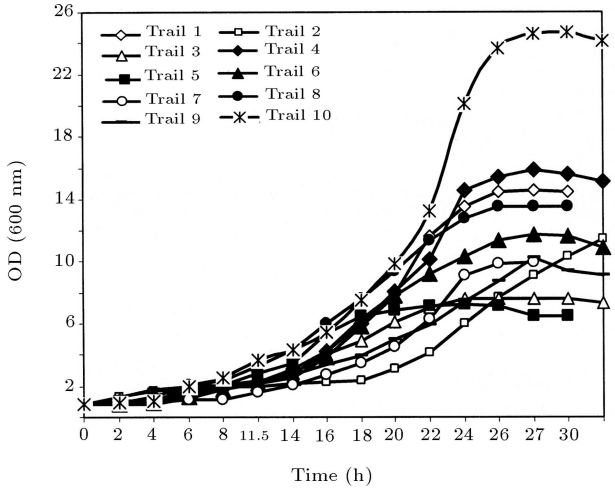

Figure 7. Optical density of Methylobacterium strain 1 in Taguchi experiments.

$$1.66\ CH_4O + 0.29\ NH_3 + 1.52\ O_2 \rightarrow CH_{1.88}O_{0.57}N_{0.29} + 0.66\ CO_2 + 2.82\ H_2O.$$

In this experiment, the biomass yield coefficient ($Y_{X/S}$) was 0.52 g/g methanol and the crude protein content was up to 75%. Under this condition, the dry biomass weight was up to 18 g/l.

The interaction of parameters in the final results is included through statistical methods and the contribution of each parameter on optimum results is shown in Figure 8. It is surprising that in this selected range of parameters, the most effective parameter is temperature. This means that accurate temperature control, at around optimum value, has a considerable effect on total biomass production, when other parameters are in the selected range. The next important parameter is seen to be the aeration rate in both sets of experiments. The more the aeration rate, the more the produced biomass until the suppressing concentration is reached. The next important parameter is seen to be the initial methanol concentration or pH. The designed levels of these two factors were narrow and converted their local optimum ranges properly. So, the effect of these parameters has less importance than the temperature and aeration rates. Despite the fact that initial methanol in global optimization was not an important parameter, it is important to note that the optimum methanol concentration and biomass yield at this value are the major factors in the economic evaluation of the industrial production of single cell protein from methanol.

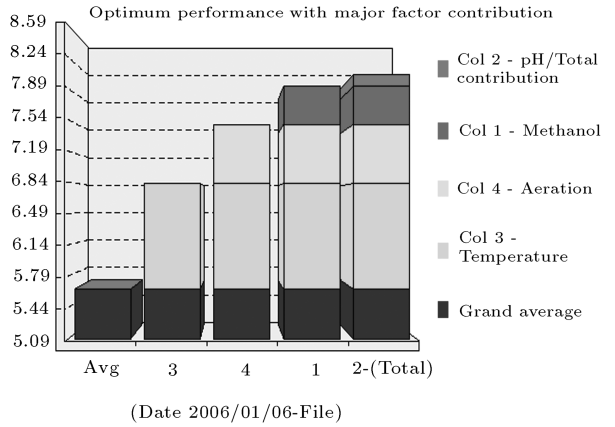

Figure 8. Contribution of parameters in the optimum results for Methylobacterium strain 1.

CONCLUSION

More than 162 species of bacteria were isolated from soil samples near the Ahwaz oil field. Some of these bacteria were characterized as methanol assimilating bacteria. Two species among these bacteria had a better yield of biomass production and were selected for the next experiments. The effects of important parameters, such as temperature, initial methanol concentration, pH and aeration rate, were studied and the trends of the growth of the two strains by a variation in these parameters and the local optimum range of each parameter, were determined. The global optimum values of these parameters were obtained by means of the Taguchi method and their contribution to the final results were determined.

By this study, several methylotrophic microorganisms from the Iranian environment were isolated and determined. These isolates showed good properties for SCP production by methanol as a substrate. The amount of dry weight gained by their growth and the total protein content were equal to SCP production world reports, as well as the high yield coefficient that was gained by the selected microorganisms. The feasibility study of this process showed the good techno-economical features of SCP production in Iran.

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