0273-2289/05/126/0205/$30.00

Effect of Hydrocarbons and Other Parameters on Hydrocarbon-Utilizing *Pichia angusta* MTCC-225

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Received March 2, 2005; Revised May 26, 2005; Accepted May 27, 2005

Abstract

Pichia angusta MTCC-225, a catalase-positive yeast that utilizes methanol and lighter hydrocarbons, is the subject of this investigation. An orthogonal experimental design L_{16} was used to investigate the effects of methanol, a gas mixture, zero air, temperature, agitation, and salts solution on hydrocarbon utilizing *P. angusta.* QUALITEK-4 Software was used for automatic design and analysis of the experimental results. Among the various parameters tested, agitation contributed the highest influence (56.5%). Zero air, methanol concentration, and gas mixture showed a moderate influence on the growth of *P. angusta*. Methanol concentration and gas mixture showed a 10.91 and 10.12% influence, respectively, on yeast growth. Zero air played an important role, with a 15.19% influence on the utilization of hydrocarbon.

Index Entries: Hydrocarbons; methanol; orthogonal array; *Pichia angusta*.

Introduction

The widespread occurrence of hydrocarbon-utilizing microorganisms in nature in both number and species attests to the wide distribution of their ability to assimilate these hydrophobic molecules. The metabolic capacity to utilize hydrocarbons is not restricted to a few microbial genera. A diverse group of bacteria and yeasts has been shown to have this ability.

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Of about 500 yeasts, 56 utilized hydrocarbons *(1)*, and almost all were in the genus *Candida* and *Pichia*. Ahearn et al. *(2)* have isolated strains of *Candida* and *Saccharomyces* that can utilize hydrocarbons. The fermentation industry has evaluated hydrocarbon-utilizing *Candida* species for commercial production of single-cell protein.

Few yeasts have the capability of metabolizing alcohols such as ethanol and methanol as their sources of carbon and energy. These yeasts are important in biotechnology for biomass propagation (e.g., *Candida utilis*) and for high-level expression of heterologous genes (e.g., *Pichia pastoris*) *(3)*. Ogata et al. *(4)* provided the first report of a eukaryotic organism (i.e., the yeast *Candida boidinii*) that utilizes methanol as its sole carbon and energy source. It is now known that methylotrophic (i.e., methanol utilizing) yeasts are found in at least four different genera: *Pichia*, *Candida*, *Hansenula*, and *Torulopsis*. Harder and Brooke *(5)*, Harder and Veenhuis *(6)*, and De Koning and Harder *(7)*, have reviewed the metabolism of methanol-utilizing yeasts. Methanol utilization provides energy for assimilation of carbon for generating yeast biomass. Methanol oxidation to CO_2 via formaldehyde provides the energy whereas fixation of formaldehyde via the xylulose monophosphate pathway provides glyceraldehyde phosphate from which cell constituents are built.

Because of the associated importance of microbes in the biotechnology industry, isolation and identification of novel microorganisms responsible for hydrocarbon utilization are important. Several reviews on ecology, physiology, and biochemistry of hydrocarbon-utilizing bacteria have been published *(8–13)*, but hydrocarbon-utilizing yeasts have not been thoroughly reviewed. Scanty reports on yeasts that focus on subjects such as the alteration of the cell surface of *Candida tropicalis* by hydrocarbon substrates *(14,15)*, application in biodegradation *(16–19)*, and use in citric acid production *(20)* and polyol production *(21–24)* are available. These studies may help in the selection of appropriate strains, determining the nutritional, metabolic, and physiological characteristics for application in petroleum microbiology, and for biotechnological applications.

The present investigation focuses on the cumulative effects on the growth of *Pichia angusta* of the variables on hydrocarbon utilization.

Materials and Methods

Microorganism and Growth Conditions

P. angusta was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, India. It was maintained on YM agar slants by subculturing at regular intervals.

Single Strength Ammonium Mineral Salts solution *(25)* was prepared by dissolving 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.7 g of K_2HPO_4, 0.54 g of KH_2PO_4, 0.5 g of NH_4Cl, 0.2 g of $CaCl_2 \cdot 2H_2O$, 4.0 mg of $FeSO_4 \cdot 7H_2O$, 0.3 mg of H_3BO_4, 0.2 mg of $CoCl_2 \cdot 6H_2O$, 0.1 mg of $ZnSO_4 \cdot 7H_2O$, 0.06 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg of $MnCl_2 \cdot 4H_2O$, 0.02 mg of $NiCl_2 \cdot 6H_2O$, and 0.01 mg of $CuCl_2 \cdot 2H_2O$

in 100 mL of distilled water at pH 7.0. Sterile methanol was added aseptically to cooled salts solution.

Three milliliters of salts solution and methanol was added to 11-mL-capacity vials according to the experimental design after the addition of inoculum (50 µL; 1.0 optical density [OD] cells). The vials were sealed with Teflon-lined butyl septa and aluminum crimp rings and partially vacuumed. To these vials a gas mixture (methane, propane, butane, *n*-butane) and zero air were added according to the experimental design. The vials were incubated at 30°C for 15 d on a shaker.

After exposure to hydrocarbons, the organism was plated on PDA and incubated at 30°C for 48 h. Colony-forming units were counted according to standard microbiological methods *(26)*.

Gas Chromatography Analyses

Gas chromatography (GC) analyses were carried out on a Nucon 5765 Gas Chromatograph equipped with a flame ionization detector. The column used was Squalance (a 1/4-in. glass-packed column with a 3-mm id and a 2-m length), and the column oven temperature was operated in isothermal mode at 40°C. Injector and detector temperatures were maintained at 80°C. Nitrogen was used as the carrier gas, and 500 µL of gas sample was extracted from the vials with a Hamilton gas-tight syringe and injected into the column. Calibration of GC was carried out using an external standard with known concentrations of methane, ethane, propane, *i*-butane, and *n*-butane. The propane in each sample was quantitatively estimated using peak area measurement with respect to standard.

Design of Experiments

An orthogonal experimental design $L_{16}(2^2 \times 3^4)$ was used to investigate the effects of methanol, gas mixture, zero air, temperature, agitation, and salts solution on hydrocarbon utilizing *P. angusta.* In the design of experiments, orthogonal means balanced, separable, not mixed or confounded. $L_a(b^c)$ is used to represent the orthogonal array (OA), in which a is the number of experimental runs, b is the number of levels for each factor or variable, and c is the number of factors investigated.

The OAs have several designs, such as OA 12, OA 18, OA 36, and OA 54, which enable a focus on the main effects and helps increase the efficiency and reproducibility of small-scale experiments. The OA is an important concept in assigning factors and interactions to the columns. OAs usually have several columns available for assignment of factors, and some columns to estimate the effect of interactions of those factors.

In the design of experiments, OAs are used to describe a large number of experimental situations mainly to reduce experimental errors and to enhance the efficiency and reproducibility of laboratory experiments. The symbolic designation of these arrays indicates the main information on the size of the experimentation; for example, L_{16} has 16 trials. The total degrees of freedom available in an OA is equal to the number of trials minus one.

Table 1
Factors and Their Levels Assigned to Different Columns

Serial no.	Factor	Level 1	Level 2	Level 3
1	Methanol (% [v/v])	1	2	3
2	Gas mixture (mL)	2	4	6
3	Temperature (°C)	30	35	—
4	Zero air (mL)	2	3	4
5	Agitation (rpm)	175	225	275
6	Salts solution (mL)	Single strength	Double strength	—

Table 2
L_{16} ($2^2 \times 3^4$) OA

Experiment no.	Methanol	Gas mixture	Temp.	Zero air	Agitation	Salts solution	CFU
1	1	1	1	1	1	1	1.8×10^6
2	1	2	1	2	2	2	2.2×10^6
3	1	3	2	3	3	1	4.1×10^6
4	1	1	2	1	1	2	2.6×10^6
5	2	1	1	1	3	2	3.4×10^6
6	2	2	1	3	1	1	1.4×10^6
7	2	3	2	2	1	2	2.6×10^6
8	2	1	2	1	2	1	3.2×10^6
9	3	1	2	2	1	1	1.6×10^6
10	3	2	2	1	3	2	4.2×10^6
11	3	3	1	1	2	1	2.8×10^6
12	3	1	1	3	1	2	5.2×10^6
13	1	1	2	3	2	2	3.6×10^6
14	1	2	2	1	1	1	2.6×10^6
15	1	3	1	1	1	2	1.8×10^6
16	1	1	1	2	3	1	4.6×10^6

Each column consists of a number of conditions depending on the levels assigned to each factor. In the present study, six different factors were used for experimentation, as shown in Table 1. The temperature and salts solutions were assigned with two levels. Hence, these two factors had eight level 1 and eight level 2 conditions (2^2), and four other factors were assigned with three levels. These four factors had eight level 1, four level 2, and four level 3 conditions (3^4). Table 2 shows the layout of the L_{16} ($2^2 \times 3^4$) OA used in the present study.

Results and Discussion

All of the combination experiments using the assigned parameter values were conducted using salts solution, gas mixture, and zero air and incubated in an orbital shaker. After 48 h of incubation, the culture broth

was plated on potato dextrose agar (PDA) and analyzed for colony-forming units (Table 2).

Colonies formed by *P. angusta* MTCC-225 were creamy, soft, moist, and butyrous on yeast extract, peptone, dextrose (YPD) agar plates. *P. angusta* MTCC-225 was a catalase-positive strain. Among naturally occurring yeasts that utilize methanol, catalase activity is an important factor. Saburo et al. *(27)* tested nine strains of methanol-utilizing yeasts belonging to the genera *Candida*, *Hansenula*, *Kloeckera*, *Pichia*, and *Torulopsis*. These strains were examined for interrelationships between their catalase content and ultrastructure. Methanol-grown cells of all the yeasts tested showed higher catalase activities than ethanol- and glucose-grown cells. In connection with this, the occurrence of a specific organelle surrounded by a single-unit membrane ("microbodies") was observed only in the methanol-grown cells. *P. angusta* MTCC-225 showed positive growth up to 6% (v/v) methanol and utilized methanol as a sole carbon source. The strain on the YPD plates showed positive growth up to a 15% methanol concentration. *P. angusta* MTCC-225 was tested for gas mixture utilization. GC analysis showed that only propane was utilized in all the tested trials. The propane-utilizing strains were more useful as standard strains in geomicrobial hydrocarbon explorations *(28,29)*.

Several bacteria, filamentous fungi, and yeasts *(8,9)* are known to utilize hydrocarbons. Most *Candida* and *Pichia* species (28) are able to assimilate methanol as a sole carbon source *(30)*. Recently, six new methanol-assimilating yeast species were reported *(31)*. It has been shown that hydrocarbon utilization is dependent on various variables such as hydrocarbon concentration, initial inoculum level, type of substrate, media composition, temperature, pH, agitation, hydrocarbon gases, interactions between the variables *(20,32,33)*, and type of microbial species. Crolla and Kennedy *(20)* reported that growth of the hydrocarbon-utilizing *Candida lipolytica* NRRL-Y-1095 is affected by the rate of inoculum, hydrocarbon concentration, and temperature. Working with *Hansenula polymorpha*, Suryadi et al. *(21)* showed that methanol concentration affected polyol yield, with the highest polyol production achieved at 1% (v/v) methanol. These studies indicate that understanding the effect of parameters is an important factor in hydrocarbon utilization.

Table 3 depicts the average effects of the factors and interactions at the assigned levels on *P. angusta* growth. The difference between the average value of each factor at level 2 and 1 indicates the relative influence of the effect; the larger the difference, the stronger the influence. The sign of the difference (+ or –) indicates whether the change from level 1 to level 2 or 3 increased or decreased the result (Table 3). Based on these data, it can be seen that temperature, agitation, and salts solution had a positive influence and the other three factors had a negative influence when they shifted from level 1 to level 2 (Table 3). However, when interactions of different factors were calculated (Table 4), interestingly, the factors perceived to be the least significant at their individual levels, temperature vs gas mixture

Table 3
Main Effects (Average Effects of Factors and Interactions) on *P. angusta* Growth

Serial no.	Factor	Level 1	Level 2	Level 3	Level 2 – level 1
1	Methanol (% [v/v])	2.912	2.650	3.449	–0.263
2	Gas mixture (mL)	3.250	2.599	2.825	–0.651
3	Temperature (°C)	2.899	3.062	—	0.162
4	Zero air (mL)	2.799	2.750	3.574	–0.049
5	Agitation (rpm)	2.449	2.949	4.074	0.500
6	Salts solution (mL)	2.742	3.200	—	0.438

Table 4
Estimated Interaction of Severity Index for Different Parameters

Serial no.	Factor	Column[a]	SI (%)[b]	Col[c]	Opt[d] Levels
1	Gas mixture × temperature	4 × 5	66.66	1	[1,1]
2	Temperature × zero air	5 × 6	57.14	3	[2,3]
3	Temperature × agitation	5 × 7	30.55	2	[2,3]
4	Methanol × salts solution	1 × 10	28.50	11	[3,2]
5	Zero air × salts solution	6 × 10	27.50	12	[3,2]
6	Agitation × salts solution	7 × 10	25.99	13	[3,1]
7	Gas mixture × zero air	4 × 6	25.83	2	[1,3]
8	Methanol × zero air	1 × 6	25.00	7	[3,3]
9	Methanol × gas mixture	1 × 4	20.53	5	[3,2]
10	Zero air × agitation	6 × 7	14.00	1	[2,3]
11	Methanol × agitation	1 × 7	10.63	6	[1,3]
12	Temperature × salts solution	5 × 10	10.41	15	[2,2]
13	Gas mixture × agitation	4 × 7	9.09	3	[2,3]
14	Gas mixture × salts solution	4 × 10	8.82	14	[1,2]
15	Methanol × temperature	1 × 5	3.90	4	[3,1]

[a]Columns = the column locations to which the interacting factors are assigned.
[b]SI = interaction severity index (100% for 90° angle between the lines, 0% for parallel lines).
[c]Col = the column that should be reserved if this interaction effect were to be studied (only 2-L factors).
[d]Opt = the factor levels desirable for optimum growth. If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without consideration of any interaction effects.

interaction, showed the highest severity index percentage (i.e., 66.66%). Similarly, the severity index percentage for gas mixture vs agitation (a stronger impact factor) was only 9.09%. These results suggest that the influence of one factor on *P. angusta* growth was dependent on the condition of the other factors.

The percentage contributions of each factor by analysis of variance (ANOVA) are shown in Table 5. The last column in Table 5 indicates the influence of each factor as a percentage. Agitation was the most significant

Table 5
Results of ANOVA

Serial no	Factor	df (*f*)	Sum of squares (*S*)	Variance (*V*)	*F* ratio (*F*)	Pure sum (*S*)	Percent (*P*[%])
1	Methanol (% [v/v])	2	1.355	0.677	33,890.8	1.355	10.916
2	Gas mixture (mL)	2	1.256	0.628	31,421.9	1.256	10.121
3	Temperature (°C)	1	0.105	0.105	5281.1	0.105	0.850
4	Zero air (mL)	2	1.886	0.943	47,171.8	1.886	15.194
5	Agitation (rpm)	2	7.046	3.523	176,171.5	7.046	56.748
6	Salts solution (mL)	1	0.765	0.765	38,281.7	0.765	6.165
	Other/error	2	–0.001	–0.001	—	—	0.006
	Total	15	12.417				100.00

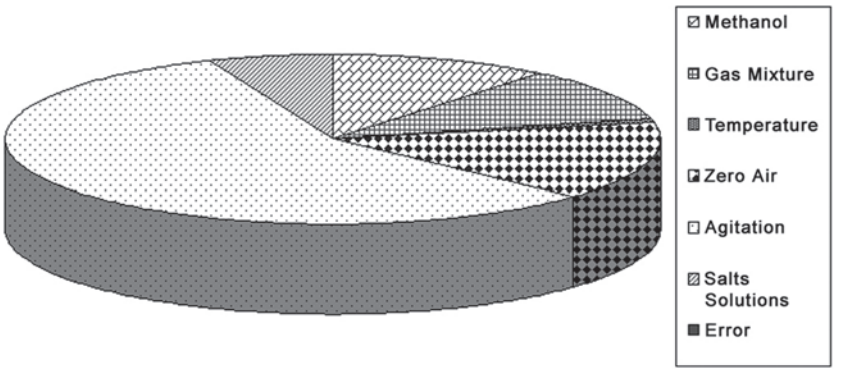

Fig. 1. Significant factors and interaction influences on growth of *P. angusta*.

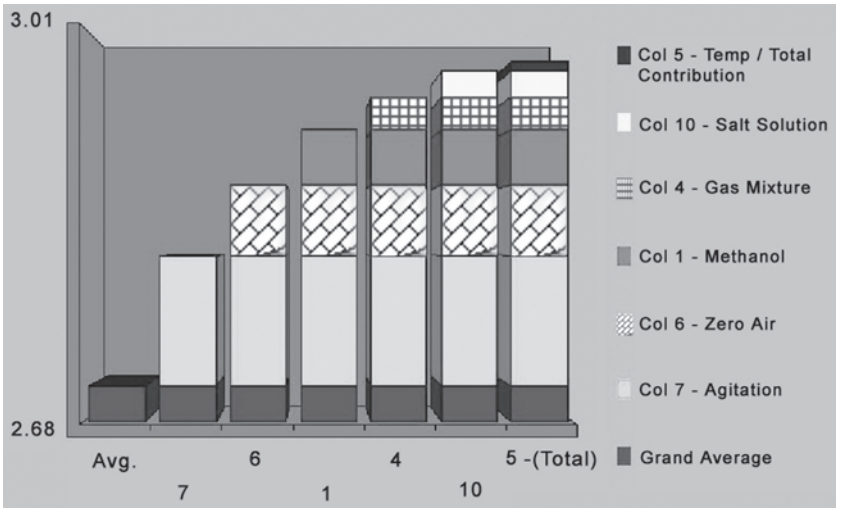

Fig. 2. Performance with major factor contributions.

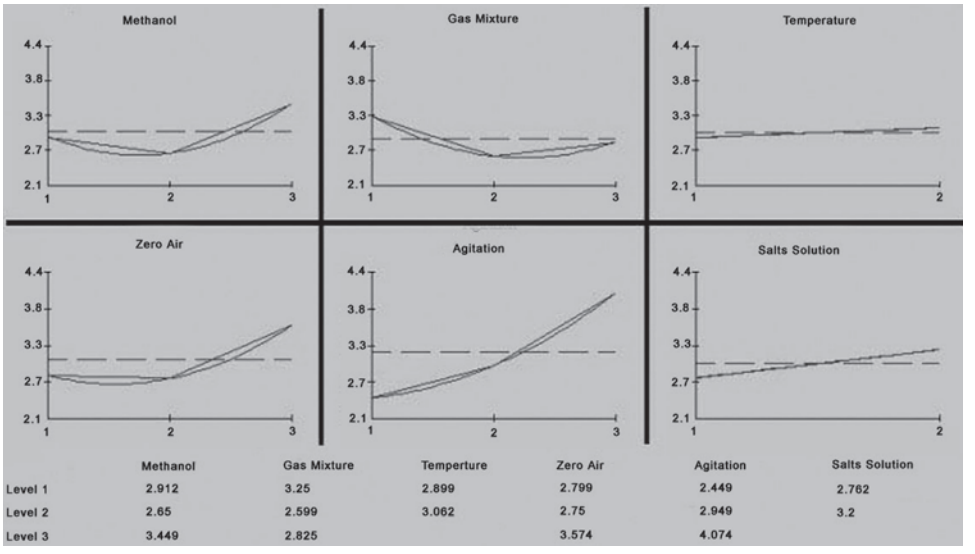

Fig. 3. Main effects of factors studied on growth of *P. angusta*.

factor for *P. angusta* growth (Fig. 1). The confidence level for the agitation factor was observed to be 99.98%. Zero air, methanol, and gas mixture (Fig. 2) showed a moderate influence on *P. angusta* growth, and salts solution showed little influence and temperature showed negligible influences on *P. angusta* growth at their individual levels (Fig. 3).

The methanol and gas mixture was included as a carbon source in this study, because methanol improves the uptake of hydrocarbon gases *(34,35)*. Bussmann et al. *(33)* demonstrated that a combination of methane, oxygen, and carbon dioxide mixing ratios in the medium influences the growth of methanotrophs.

Conclusion

A combination of the factors methanol, hydrocarbon gas mixture, zero air, temperature, agitation, and salts solution and their levels involved in *P. angusta* growth were identified. The design of experiments using the L_{16} OA is useful to study the effect of different factors on *P. angusta* growth. The present analysis suggests that zero air, methanol, and gas mixture had a moderate influence whereas agitation had a strong influence on *P. angusta* growth. The remaining factors, salts solution and temperature, were of little significance at their individual levels.

Acknowledgments

We are grateful to Dr. V. P. Dimiri, director, National Geophysical Research Institute, for granting permission to publish this work and taking keen interest in setting up the microbiology laboratory at National Geophysical Research Institute, Hyderabad, India. We also acknowledge financial support from Oil Industry Development Board, New Delhi, India.

References

1. Komagata, K., Nakase, T., and Katsuya, N. (1964), *J. Gen. Appl. Microbiol.* **10,** 313–321.
2. Ahearn, D. G., Meyers, S. P., and Standard, P. G. (1971), *Dev. Ind. Microbiol.* **12,** 126–134.
3. Walker, G. M. (1998), in *Yeast Physiology and Biotechnology* (Walker, G. M., ed.), John Wiley & Sons, New York, pp. 231–233.
4. Ogata, K., Nishikawa, H., and Ohsugi, M. (1969), *Agric. Biol. Chem.* **33,** 1519, 1520.
5. Harder, W. and Brooke, A. G. (1990), in *Yeast: Biotechnology and Biocatalysis* (Verachtert, H. and Dermot, R., eds.), Marcel Dekker, New York, pp. 395–428.
6. Harder, W. and Veenhuis, M. (1989), in *The Yeasts*, 2nd ed., vol 3 (Rose, A. H. and Harrison, J. S., eds.), Academic, London, pp. 289–316.
7. De Koning, W. and Harder, W. (1992), in *Methane and Methanol Utilizers* (Murrel, J. C. and Dalton, H., eds.), Plenum, New York, pp. 207–244.
8. Ronald, M. A. (1981), *Microbiol. Rev.* **45,** 180–209.
9. Joseph, G. L. and Rita, R. C. (1990), *Microbiol. Rev.* **54,** 305–315.
10. Richard, S. H. and Thomas, E. H. (1996), *Microbiol. Rev.* **60,** 439–471.
11. Anthony, C. (1982), in *The Biochemistry of Methylotrophs*, Academic, London, pp. 1–41.
12. Samir, S. R. and Naser, A. S. (1993), *Adv. Appl. Microbiol.* **39,** 29–90.
13. Ronald, W. K. (1993), in *Soil Gas and Related Methods for Natural Resource Exploration*, vol. 4 (Neidleman, S. and Laskin, A. I., eds.), John Wiley & Sons, West Sussex, England, pp. 61–83.

14. Kappelli, O. and Fiechter, A. (1977), *J. Bacteriol.* **131,** 917–921.
15. Kappelli, O., Muller, M., and Fiechter, A. (1978), *J. Bacteriol.* **133,** 952–958.
16. Oh, Y. S., Maeng, J., and Kim, S. J. (2000), *Appl. Microbiol. Biotechnol*. **54,** 418–423.
17. Ismailov, N. M. (1985), *Mikrobiolgiia* **54,** 668–674.
18. Chaillan, F., Fleche, A. L., Bury, E., et al. (2004), *Res. Microbiol*. **155,** 587–595.
19. Kim, H. S., Jeon, J. W., Kim, S. B., Oh, H. M., Kwon, T. J., and Yoon, B. D. (2002), *Biotechnol. Lett*. **24,** 1637–1641.
20. Crolla, A. and Kennedy, K. J. (2001), *J. Biotechnol*. **89,** 27–40.
21. Suryadi, H., Katsuragi, T., Yoshida, N., Suzuki, S., and Tani, Y. (2000), *J. Biosci. Bioeng*. **3,** 236–240.
22. Vongsuvanlert, V. and Tani, Y. (1989), *J. Ferment. Bioeng*. **67,** 35–39.
23. Tani, Y. and Vongsuvanlert, V. (1987), *J. Ferment. Technol*. **65,** 405–411.
24. Vongsuvanlert, V. and Tani, Y. (1988), *J. Ferment. Technol*. **66,** 517–523.
25. Ronald, M. A. and Lawrence, C. P. (1996), in *Handbook of Microbiological Media* (Parks, L. C., ed.), CRC Press, New York, pp. 79–81.
26. Collins, C. H., Lyne, P. M., and Grange, J. M. (1995), in *Microbiological Methods*, Butterworth-Heinemann, Oxford, UK, pp. 151–153.
27. Saburo, F., Atsuo, T., Susumu, K., Shigeki, Y., Yutaka, T., and Masako, O. (1975), *J. Bacteriol.* **123,** 317–328.
28. Singh, N., Kapoor, S., Jain, M. A., Kumar, A., and Koshel, K. C. (1999), in *Proceedings of the Third International Petroleum Conference & Exhibition*, PETROTECH-99 (Bhatnagar, A. K., ed.), Thomson Press (I), Faridabad-Haryana, India, pp. 323–326.
29. Steven, A. T. (1995), in *Surface Geochemistry in Petroleum Exploration* (Tedesco, S. A., ed.), Springer, New York, pp. 132–143.
30. Middelhoven, W. J. and Kurtzman, C. P. (2003), *Anton van Leeuwenhoek* **83,** 69–74.
31. Peter, G., Tornai-Lehoczki, T., Fulop, L., and Dlauchy, D. (2003), *Anton van Leeuwenhoek* **84,** 147–159.
32. Martin, A. and Sivagurunathan, M. (2003), *Commun. Agric. Appl. Biol. Sci*. **68,** 175–178.
33. Bussmann, I., Pester, M., Brune, A., and Schink, B. (2004), *FEMS Microbiol. Ecol*. **47,** 179–189.
34. Jensen, S., Prieme, A., and Bakken, L. (1998), *Appl. Environ. Microbiol*. **64,** 1143–1146.
35. Benstead, J., King, G. M., and Williams, H. G. (1998), *Appl. Environ. Microbiol.* **64,** 1091–1098.