0273-2289/03/105-108/0247/$20.00

Saccharification of Marine Microalgae Using Marine Bacteria for Ethanol Production

Mitsufumi Matsumoto,[*,1,3] Hiroko Yokouchi,[1] Nobukazu Suzuki,[2] Hiroshi Ohata,[3] and Tadashi Matsunaga[1]

[1]Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-26, Naka-cho, Koganei, Tokyo, Japan, E-mail: mitsufumi-matsumoto@jpower.co.jp; [2]Chemical and Insulation Technology Group, Power and Industrial Systems R&D Center, Toshiba Co. Ltd., Tsurumi-ku, Yokohama, 230-0045, Japan; and [3]New Energy and Technology Development Department, Electric Power Development Co. Ltd., Chuo-ku, 104-8165, Tokyo, Japan

Abstract

The saccharification of marine microalgae using amylase from marine bacteria in saline conditions was investigated. An amylase-producing bacterium, *Pseudoalterimonas undina* NKMB 0074 was isolated and identified. The green microalga NKG 120701 was determined to have the highest concentration of intracellular carbohydrate and was found from our algal culture stocks. *P. undina* NKMB 0074 was inoculated into suspensions containing NKG 120701 cells and increasingly reduced suspended sugars with incubation time. Terrestrial amylase and glucoamylase were inactive in saline suspension. Therefore, marine amylase is necessary in saline conditions for successful saccharification of marine microalgae.

Index Entries: Saccharification; marine algae; marine bacteria; amylase; ethanol; biomass.

Introduction

Marine microalgae living in marine environments have been studied for biologic production of useful material such as fatty acids *(1,2)*, bioactive compounds *(3–5)*, hydrogen *(6,7)*, and polysaccharides *(8)* for three decades *(9)*. Marine microalgae can produce these materials using only solar conversion and CO_2 *(10–12)*.

*Author to whom all correspondence and reprint requests should be addressed.

Application of high carbohydrate–producing marine microalgae can generate an alternative biomass resource for ethanol production. So far, biomass resources for ethanol production commonly have been sugarcane and sweet corn *(13)*. As other biomass resources, wood chips *(14)*, xylose as sugar sources *(15–17)*, culled apple juice *(18)*, and raw wheat flour *(19)* has been used.

The production of ethanol by fermentation from biomass consisting of carbohydrates was composed of two processes such as saccharification and fermentation. Saccharification requires enzymes to hydrolyze carbohydrates prior to fermentation. It is primarily performed using terrestrially derived amylase for ethanol production *(20,21)*. The utilization of marine biomass would require desalinization if a terrestrially sourced enzyme were to be used. Therefore, development of a system utilizing amylase from a marine source would be beneficial. In the present study, we investigated the saccharification of marine microalgae using amylase derived from a marine bacteria.

Materials and Methods

Cell Culture

Marine bacteria were maintained in marine YS medium plates containing 10 g/L of potato starch and 1 g/L of yeast extract in artificial seawater. The artificial seawater was purchased from Senjyu. Marine microalgae were maintained in BG-11 medium with 3% NaCl *(22)* under a light intensity of 40 μE/(m^2·s).

Isolation and Screening of Amylase Producing Marine Bacteria

Samples of sand, mud, and sea grass from the Japanese coastline (Tateyama, Chiba) were collected. Samples were diluted with sterilized artificial seawater. Aliquots were inoculated on minimum medium plates (10 g/L of starch in artificial seawater). The plates were incubated for 2 d at room temperature (24–26°C). Colonies were picked up and transferred to marine YS medium. Pure strains of the bacteria were isolated by repeated plating. The bacteria strain was determined by 16S rDNA sequence analysis.

Marine bacteria were inoculated at room temperature into L glass tubes containing 10 mL of marine YS medium (OD_{660}: 0.05) and shaken at 100 rpm. Culture suspensions (1 mL) were harvested after 24 and 48 h. The cells were removed by centrifuging at 8,000*g* for 5 min, and the supernatants were measured for glucose using an enzyme-based F-kit (Boehringer).

Amylase Activity of Marine Bacteria

Amylase activity was determined by measuring reduced sugars from starch. The assay mixture contained 0.7 mL of 1.0% soluble starch in artificial seawater and 0.3 mL of cell-free culture supernatant. The assay solution (n = 3) was incubated for 5 min at room temperature. Reduced sugars were

determined by the dinitrosalicylic acid method using glucose as the standard *(23)*. One unit of activity is defined as the amount of enzyme per milliliter that produces 1 µmol of reduced sugars from starch in 1 min.

Cell growth was monitored by OD_{660}. Protein production was detected by the Lowry method *(24)*. The protein profiles in YS medium were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver.

Screening of High Starch–Containing Marine Microalgae

Marine microalgae were cultured in aerated 500-mL hexagonal flasks with illumination (40 µE/[m^2·s]) at room temperature. The carbohydrate content from 76 strains was determined using the phenol-sulfuric acid method. Dry cells of 0.5 mg (n = 3) were used and evaluated as reduced sugars per gram of dry cell.

Saccharification Using Marine Bacteria and Bioethanol Production

Fresh algal cells of 1.82 g wet wt were centrifuged and resuspended in 10 mL of marine YS medium without starch. Cells were mechanically broken using a vortex containing silica beads. The algal residues were autoclaved at 121°C for 10 min. Marine bacteria (0.14 g wet wt) were inoculated into 10 mL of this solution and incubated with shaking (100 rpm) at room temperature. The samples (n = 3) were harvested at 12 and 24h, and the reduced sugars in culture suspension was measured by the method mentioned previously.

Results

Glucose Production from Starch by Marine Bacteria

The bacteria of 191 strains were isolated from the marine environment. The isolated strain NKMB 0074 showed the highest glucose concentration (Table 1). Most of the tested strains had little glucose concentration. The glucose concentration of NKMB 0074 after 24 h of incubation was 0.59 g/L and reached 1.85 g/L after 48 h.

Using 16S rDNA sequence analysis, NKMB0074 was determined to be in the *Pseudoalteronomas* genus with 99% homology. The species was determined by 16S rDNA phylogenic analysis and was identified as *Pseudoalteronomas undina*.

Amylase Activity of Marine Bacteria

Amylase activity of *P. undina* NKBG 0074 was defined in artificial seawater. After 1 day of incubation, amylase activity was 51.18 ± 4.35 U/mg of protein (Table 2). The α-amylase of *Porcine pancreas* and glucoamylase of *Rhizopus sp*. were used for comparative study. The two enzymes were found to be inactive in artificial seawater. Marine amylolytic enzymes were preserved in saline conditions and could therefore be used for saccharification of marine algae.

Table 1
Glucose Concentration in Culture Supernatant of Marine Bacteria

Strain	Glucose concentration (mg/L)[a] 1 d	2 d
NKMB 0006	0.86	698.1
NKMB 0018	ND	61.3
NKMB 0019	ND	15.6
NKMB 0021	2.6	10.4
NKMB 0064	8.6	8.6
NKMB 0066	14.0	10.4
NKMB 0069	1.7	19.1
NKMB 0072	ND	65.7
NKMB 0074	592.7	1855.9
NKMB 0085	ND	25.9
NKMB 0087	6.0	494.2
NKMB 0091	40.6	16.4
NKMB 0093	605.0	1131.8
NKMB 00101	58.8	631.6
NKMB 00106	125.3	875.2
NKMB 00130	150.6	94.2
NKMB 00144	ND	186.6
Others: 173 strains	ND	ND or >8.6
Total strains = 190		

[a]ND, not determined

Cell Growth, Glucose Accumulation and Specific Protein Production

The growth of *P. undina* NKMB0074, glucose accumulation, and protein profiles in YS medium was analyzed. Glucose accumulation in the supernatant started after 18 h of incubation, and cell growth reached stationary growth after 24 h (Fig. 1). Glucose accumulation started at the beginning of stationary growth. This result was expected that glucose consumption of bacteria cell was decreased. Therefore, the glucose concentration in suspension was increased. The final glucose concentration reached 2.3 g/L after 48 h of incubation.

Protein concentrations in the supernatant were determined to increase from 18 h of incubation, the same as the onset of glucose accumulation (Fig. 2). Total protein concentrations were up to 1 g/L at the end of incubation and were related to glucose accumulation.

The protein profile in YS medium was investigated by SDS-PAGE, and the production of a specific protein (86 kDa) was observed (Fig. 3). Furthermore, only the 86-kDa protein increased with incubation time.

Table 2
Enzyme Activity of *P. undina* NKMB0074 in Artificial Seawater[a]

Sample	Amylase activity (U[b]/mg protein)
Culture supernatants	51.18 ± 4.35
α-Amylase[c]	0.59 ± 0.02
Glucoamylase[d]	0.94 ± 0.04

[a]Suspension at 1 d of culture was used.
[b]One unit of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugars/(min·mL) of culture suspension.
[c]α-Amylase was obtained from *P. pancreas* and activity showed 20,500 U/mg of protein.
[d]Glucoamylase was obtained from *Rhizopus* sp. and showed 38.1 U/mg of protein.

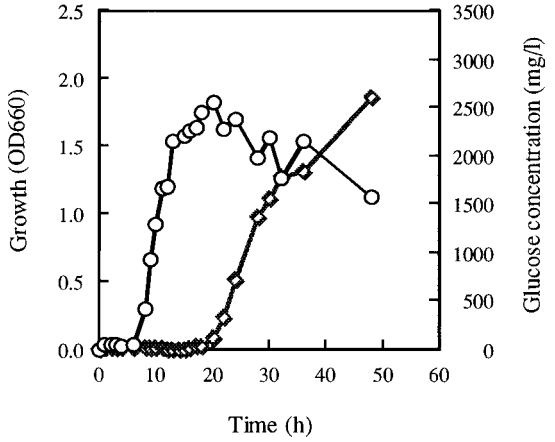

Fig. 1. Time course of growth of *P. undina* NKB 0074 and glucose concentration in medium. (○)Growth; (◇) glucose concentration.

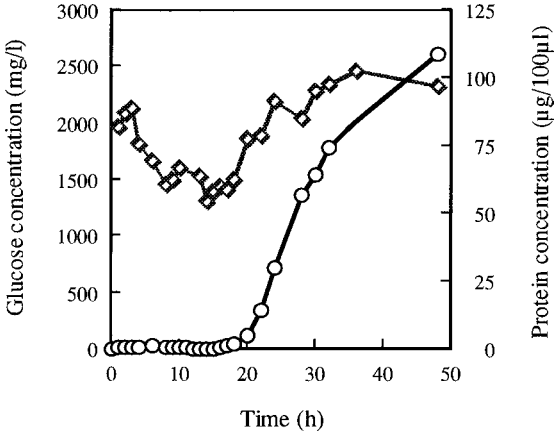

Fig. 2. Time courses of glucose and protein concentrations in medium when *P. undina* NKBG0074 was incubated for 48 h. (○)Glucose concentration; (◇) protein concentration.

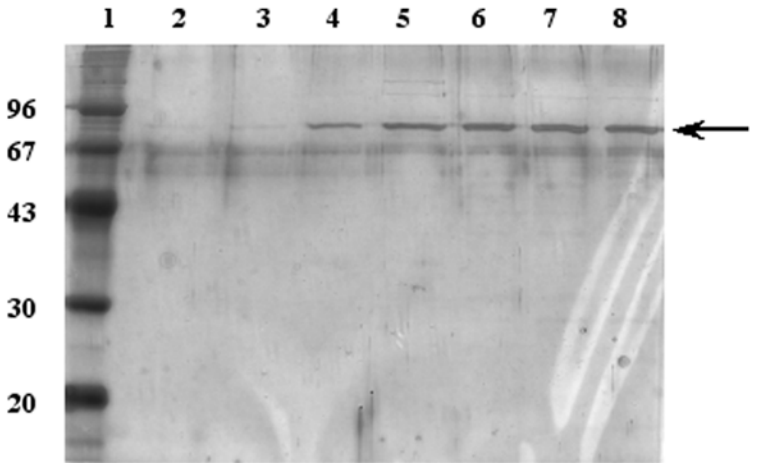

Fig. 3. Extracellular protein analysis by SDS-PAGE of different incubation times. Lane 1, standard; lane 2, 1 h; lane 3, 6 h; lane 4, 12 h; lane 5, 18 h; lane 6, 24 h; lane 7, 36 h; lane 8, 48h. Twenty micrograms of protein of was applied.

Saccharification of Marine Microalgae Biomass

The reduced sugars from 76 marine microalgae are given in Table 3. The green alga NKG 121701 strain was found to contain the highest reduced sugars, totaling >50% of dry cell weight. The other strains were <40%. Therefore, the green alga NKG 121701 was used for further work.

P. undina NKMB0074 was inoculated into suspensions containing NKG 120701 cells. The reduced sugars in suspension increased with increasing time of incubation (Table 4). After 24 and 48 h of incubation, reduced sugars were determined at concentrations of 0.9 ± 0.28 and 2.82 ± 0.62 mg/mL. After 48 h, there was a 38% reduction in microalgae biomass, correlating to a 1.82–1.13 g in 10 mL. Of this, 4.1% of NKG 120701 biomass was converted to reduced sugars. Saccharification of potato starch and microalgae using terrestrial amylase and glucoamylase was also tested. However, no saccharification was observed in saline conditions (Table 4).

Discussion

The utilization of renewable resources for energy and chemicals is expected to increase in the near future. Ethanol can be produced by microbial fermentation from renewable plant sources. Carbohydrates containing starch and polysaccharides, and lignocellulosic materials containing cellulose, hemicellulose, and lignin, are the most abundant renewable organic resources. Much attention has therefore been focused on genetically engineering strains that can efficiently utilize carbohydrates and lignocellulosic materials and convert them into useful compounds such as ethanol *(25)*.

Table 3
Reduced Sugar Amounts of Marine Microalgae

Strain	Reduced sugar amounts (Mg/g dry wt)	Content (%)
Green alga		
NKG 040502	419.7 ± 73.5	41.9
NKG 040502	419.7 ± 73.5	41.9
NKG 042501	425.6 ± 58.0	42.5
NKG 041304	429.7 ± 45.2	42.9
NKG 041102	453.2 ± 37.2	45.2
NKG 042905b	451.8 ± 103.1	45.1
NKG 121701	531.3 ± 27.0	53.1
NKG 132301	442.9 ± 76.1	44.2
Others: 68 strains	>40	>40
Total strains = 76		

Table 4
Bioconversion to Reduced Sugars of Marine Microalgae Using Several Amylase

	Reduced sugars concentration (mg/mL)[a]	
Enyme	1 d	2 d
Amylase of *P.undina* NKMB 0074	0.90 ± 0.28	2.82 ± 0.62
Amylase of *P. pancreas*[c]	ND	ND
Glucoamylase of *Rhizopus* sp.[c]	ND	ND

[a]ND, not determined
[b]Alive cells of *P. undina* NKMB 0074 were inoculated at a concentration of 0.14 g/10 mL.
[c]Each enzyme was used at a concentration of 1 mg of protein/mL.

Renewable marine resources including macro- and microalgae could be applied to convert to energy and chemical compounds. Previous work, reported that as applications of renewable marine resources, methane production using marine microalgae biomass *(26)* and a method utilizing floating ceramic supports for the cultivation of marine microalgae have been developed to take advantage of this vast resource *(27)*. Application utilizing a large surface area of an ocean can produce vast amounts of marine biomass and useful materials.

The microbial production of ethanol from carbohydrates is mainly dependant on saccharification. Amylase produced from *P. undina* NKMB0074 converted the biomass of the marine green alga NKG 121701 to reduced sugars. Terrestrial amylase activity was not observed under saline conditions, indicating that it cannot be used industrially for the saccharification of marine microalgae. The inactivity of these enzymes

resulted mainly from effects of salinity. Although saccharification of marine microalgae biomass using amylase of the marine bacterium *P. undina* NKMB 0074 was observed, enzyme production of amylase from *P. undina* NKMB 0074 was only about 1 g/L, and, therefore, the conversion efficiency to reduced sugars was low (4.1%). To increase conversion efficiency, the application of other glucoamylases and proteases may preserve activity under the saline conditions needed. Future work will investigate the optimum conditions required for saccharification using marine amylase and microalgae for ethanol production.

References

1. Burgess, J. G., Iwamoto, K., Miura, Y., TaKano, H., and Matsunaga, T. (1993), *Appl. Microbiol. Biotechnol.* **39,** 456–459.
2. Miura, Y., Sode, K., Nakamura, N., Matsunaga, N., and Matsunaga, T. (1993), *FEMS Microbiol. Lett.* **107,** 163–168.
3. Wachi, Y., Burgess, J. G., Takahashi, J., Nakamura, N., and Matsunaga, T. (1995), *J. Biotechnol.* **2,** 210–213.
4. Wake, H., Akasaka, A., Umetsu, H., Ozeki, Y., Shimomura, K., and Matsunaga, T. (1992), *Plant Cell Rep.* **11,** 62–65.
5. Miura, Y., Sode, K., Nakamura, N., and Matsunaga, T. (1993), *J. Mar. Biotechnol.* **1,** 134–146.
6. Kumazawa, S. and Mitsui A. (1981), *Int. J. Hydrogen Energy* **6,** 339–348.
7. Nandi, R. and Sengupta S. (1998), *Crit. Rev. Microbiol.* **24,** 61–84.
8. Sudo, H., Burgess, J. G., Takemasa, H., Nakamura, N., and Matsunaga, T. (1995), *Curr. Microbiol.* **30,** 219–222.
9. Mitsui, A. (1975), in *Proceedings of the 3rd Ineternational Ocean Development Conference*.
10. Borowitzka, L.J. and Borowitzka M.A. (1989), in *Industrial Production: Method and Economics*. Cresswell, R. C., Rees, T. A. V., and Shah, N., eds., Elsevier Applied Science, London, UK, pp. 294–316.
11. Borowitzka, M. A. (1992), *J. Appl. Phycol.* 4, 267–279.
12. Patterson, G. M. L. (1996), *J. Sci. Ind. Res.* **55,** 669–684.
13. Mielenz, J. R. (2001), *Curr. Opin. Microbiol.* **4,** 324–329.
14. Razmovski, R. and Pejin D. (1996), *Folia Microbiol.* **41,** 201–207.
15. Joachimsthal, E. and Rogers P. (2000), *Appl. Biochem. Biotechnol.* **84/86,** 343–356.
16. Krishhan, M., Blanco, M., Shattuck, C. K., Nghiem, N. P., and Davison, B. H. (2000), Appl. Biochem. Biotechnol. **84/86,** 525–541.
17. Lawford, H. and Rousseau, J. (2000), *Appl. Biochem. Biotechnol.* **84/86,** 277–293.
18. Sandhu, D. and Joshi V. (1994), *Indian J. Exp. Biol.* **32,** 873–876.
19. Montesinos, T. and Navarro, J.-M. (2000), *Enzyme Microbiol. Technol.* **27,** 362–370.
20. Abouzied, M. M. and Reddy, A. C. (1986), *Appl. Environ. Microbiol.* **52,** 1055–1059.
21. Fumihisa, K., Sawada, T., Nakamura, Y., Ohnaga, M., Godliving, M., and Ushiyama, T. (1998), *Appl. Biochem. Biotechnol.* **69,** 177–189.
22. Rippka, R., Deruelles, J., Watherbury, J. B., Herdman, M., and Stanier, R. Y. (1979), *J. Gen. Microbiol.* **111,** 1–61.
23. Miller, G. L.(1959), *Anal. Chem.* **31,** 426–428.
24. Lowry, O., Rosebrough, N. J., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
25. Aristos, A. and Merja, P. (2000), *Curr. Opin. Biotechnol.* **11,** 187–198.
26. Matsunaga, T. and Izumida, H. (1984), *Biotechnol. Bioeng. Symp.* **14,** 407–418.
27. Matsumoto, M., Yoshida, E., Takeyama, H., and Matsunaga, T.(2000), *Appl. Biochem. Biotech.* **84/86,** 51–57.