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Production of extracellular polysaccharide by *Monilinia fructigena* for aquaculture

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Production of extracellular polysaccharide by *Monilinia fructigena* in B-I medium containing cereals was higher than that in glucose medium. Productivities in B-I medium and glucose medium were 0.7 g/ℓ and 0.2~0.3 g/ℓ, respectively. The maximum content of polysaccharide occurred at the rising point from the lowest pH of culture. As the apparent viscosity of the polysaccharide solution increased, the flow Index (m) decreased, and the consistency Index (Kc) also increased. The polysaccharide solution was a typical pseudoplastic fluid. The mycelium was separated from the culture solution by 300 μm mesh-filter and the polysaccharide was precipitated by adding 50% of ethanol (v/v). The amount of the polysaccharide removed from the filtrated solution was 0.45 g/ℓ and the amount adhered to the mycelium was 0.25 g/ℓ. In experiments for investigating growth enhancement of rotifer (*Brachionus plicatilis*) by the polysaccharide, the dose of the polysaccharide was 1 mg per 10,000 organisms of rotifer. Maximum specific growth rate of rotifer with feed consisting of sea *Chlorella* sp. and the polysaccharide was 1.095/day in the batch culture for 10 days. A semi-continuous culture was done for 30 days, the biomass of rotifer could be harvested twice. Maximum specific growth rate with sea *Chlorella* sp. and the polysaccharide was 0.734/day before the first harvest, and 1.685/day before the second harvest. Productivity was 38 cells/mℓ · day with sea *Chlorella* sp. and the polysaccharide.

Key words: *Monilinia fructigena*, extracellular polysaccharide, *Brachionus plicatilis*

Introduction

β-Glucan is the component of some cereals (Fincher et al., 1975) and yeast's cell wall (Machova et al., 1995) and a kind of β-glucan was composed with a β-1,3-glucan having single β-1,6-glucosyl side chains on every second glucose unit (Santamaria et al., 1978). Especially β-1,3-D-glucans from the cell wall of *Saccharomyces cerevisiae* are classified as a nutrition product with GRAS (Generally Recognized As Safe) classification by the FDA. They could stimulate the activity of Macrophage (Mϕ), regulate the aging caused by ultraviolet light, scavenge the free-radical in human body (Browder et al., 1990; Williams et al., 1996). β-1,3 / 1,6-glucan from cell wall of *Saccharomyces cerevisiae*, which was convinced of immunity and capabilities of nutrients and dietary foods, was applied to aquaculture and dietary fiber (Engstad et al., 1992; Rorstad et al., 1993; Sung et al., 1994).

In 1959, one of the first extracellular polysaccharide by fungi was isolated from sclerotia of *Monilinia libertiana* and was constructed to be a β-1,3-linked glucan with single glucose residues attached at intervals by β-1,6-linkages by Kitahara & Takeuchi. In 1963, the structure of a similar polysaccharide produced by Sclerotium rolfsii was investigated by Johnson et al. and then analogous polysaccharides have been separated from *Claviceps purpurea* (Perlin & Taber, 1963), *Pullularia pullulans* (Bouveng et al., 1963), *Claviceps fusiformis* (Buck et al., 1968) and *Schizophyllum commune* (Kikumoto et al., 1970 a,b).

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But the studies for the increment of yield with fungi that produced extracellular polysaccharide were not enough to apply optimized conditions to scaled-up system. In this publication, new culture medium for the production of extracellular polysaccharide and decrement of production cost, was investigated in a scaled-up of 19 ℓ-bioreactor. In addition, for the nutritional verification of β-glucan by *Monilinia fructigena*, the polysaccharide was applied to rotifer (*Brachionus plicatilis*) and was fed for fry, to compare with 2 representative feeds like sea *Chlorella* sp. and the yeast.

Materials & Methods

Strain & Medium

Monilinia fructigena in this work was kindly donated from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and was cultivated on the PDA plate (Potato Dextrose Agar, DIFCO). After mycelium was spreading, the growing mycelium was cut from the plate (1×1 cm). The cut mycelium was used as an inoculum to the preculture in the cultivating broth composed of glucose medium with yeast extract (1g/ℓ) at 25℃, and 100rpm for 7 days (Reyes & Byrde, 1973). Glucose medium and B-I medium in our laboratory were sterilized at 121℃, for 15 min.

Culture conditions for production of polysaccharide

Culture conditions were 15 ℓ working volume, 25℃ cultivation temp., 250~300 rpm agitation speed, 1.33% (v/v) initial inoculation concentration, and 0.133~0.345 vvm air supply rate. A schematic diagram of 19 ℓ-bioreactor is shown in Fig. 1. For removing contaminants during sampling, a steam generator was used to sterilize the sampling part.

Partial purification of polysaccharide

The partial purification of polysaccharide was carried out due to washing and precipitating by adding ethanol and acetone (30%). A schematic diagram of the purification process is shown in Fig. 2. In the case of part A, 1 ℓ filtrated solution from culture was stirred with 500 mℓ ethanol for 0.5~1.0 hr. Then polysaccharide was separated from polysaccharide suspension and was redissolved with double distilled water. After agitating on the magnetic stirrer for 0.5~1.0 hr, polysaccharide was reprecipitated by adding an half volume of ethanol. The polysaccharide was redissolved with double distilled water. In the last step, for removing

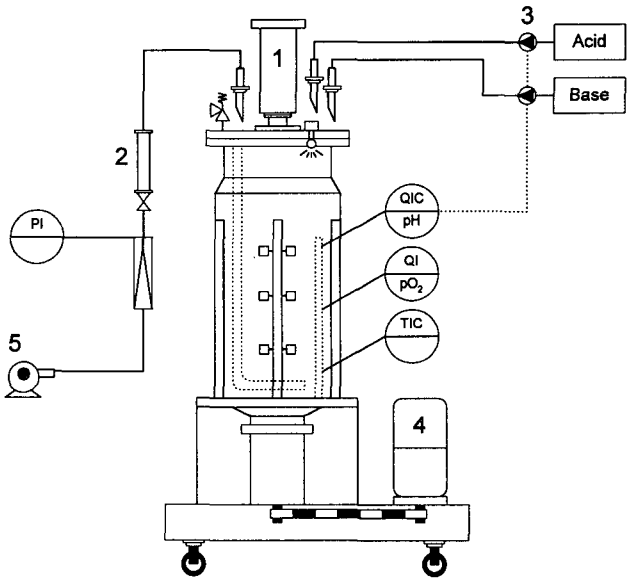

Fig. 1. Schematic of 19 ℓ-bioreactor (1 : Out-let air filter, 2 : In-let ait filter, 3 : Peristaltic pump, 4 : Agitation motor, QIC/pH : Quantity Indicator and Controller of pH, QI/pO_2 : Quantity Indicator of pO_2, TIC : Temperature Indicator and Controller, PI : Pressure Indicator).

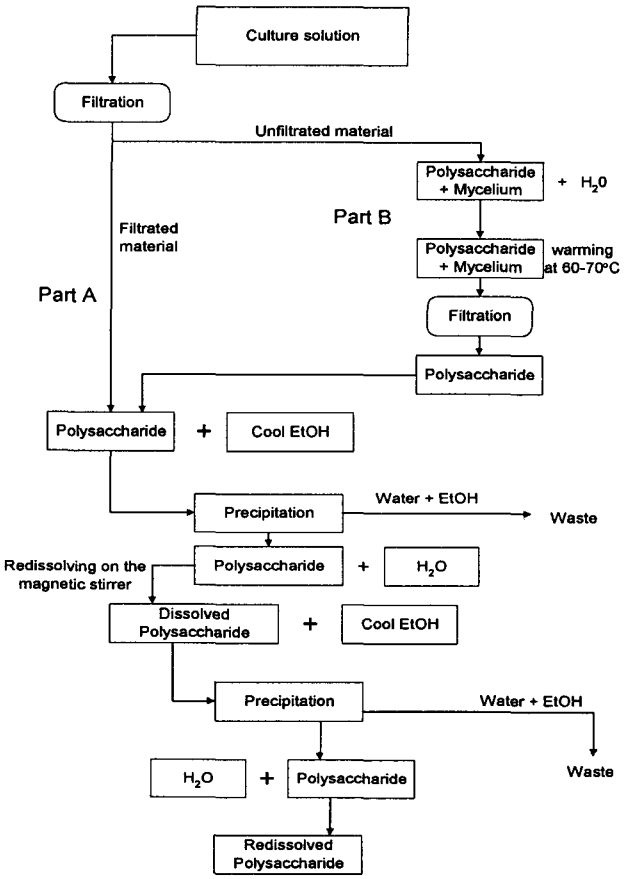

Fig. 2. Schematic diagram of partial purification process with organic solvent.

polysaccharide that was not redissolved in the purification process, polysaccharide suspension was filtered with 300 μm filter again. In the case of part B, mycelium complex was mixed with the same volume of water, then heated and agitated on the magnetic stirrer for 1~2 hrs and filtered with 300 μm filter. Then the filtrates were purified in Part A.

Application of polysaccharide to rotifer

To establish the effect of extracellular polysaccharide produced by *Monilinia fructigena*, it was applied to rotifer (*Brachionus plicatilis*), as a feed for fry, to compare with 2 representative feed, sea *Chlorella* sp. and the yeast which was produced with baker's yeast and some nutritious materials.

Rotifer was cultured under sun light at 25℃. Initial concentration of rotifer was 20 cell/mℓ in each feed conditions. Productivities of rotifer with 4 different types of feed were observed in batch and semi-continuous culture. Feeding conditions were followed: I, sea *Chlorella* sp.; II, sea *Chlorella* sp. and the polysaccharide; III, sea *Chlorella* sp. and the yeast; and IV, sea *Chlorella* sp., the polysaccharide and the yeast. Sea *Chlorella* sp. was supplied as 106 cell per a cell of rotifer, the polysaccharide and the yeast were supplied as 1 mg and 10 mg per 10,000 cells, respectively. Rotifer and sea *Chlorella* sp. were obtained from Jin-Nam Aquaculture and they were cultivated with artificial sea water for a month in the laboratory. The yeast was obtained from Yi-Hwa Co. Ltd. The artificial sea water was used as described in Schloesser (1994).

Cultivation process of rotifer

· Batch culture

The growth curves and their μ_{max} were calculated in 4 different conditions that supplied to rotifer with the same amount. The harvesting points (stationary phase in batch culture) were applied to next semi-continuous culture.

· Semi-continuous culture

The semi-continuous culture was performed under the same conditions as in the batch culture. Rotifer was harvested in stationary phase to obtain productivity and control the number of rotifer as the initial number. After that, only sea *Chlorella* sp. was supplied as a feed for rotifer by the ratio of 10^5 cells/mℓ per 1 rotifer.

Experimental analysis

· After the mycelium suspension was filtered with 300 μm filter and the mycelium was agitated to remove adhesive polysaccharide on the magnetic stirrer by adding 5 times distilled water, the biomass of *Monilinia fructigena* was calculated from the basis dried at 60℃ for 12 hrs. The content of the polysaccharide was measured after centrifuge of the mycelium suspension (2000×g, 10min; Archer et al., 1977), the precipitate was washed and purified with ethanol and water (3 times), then dried at 60℃ for 12 hrs.

· The apparent viscosity of the culture broth was measured according to the cultivation time by means of brookfield viscometer (Brookfield Engineering Labs, Inc) and the characteristics of the fluid were determined though the consistency Index (Kc) and the flow Index (m). Then the dry-basis transformation of polysaccharide according to increasing viscosity was studied. · Moisture, crude protein, crude lipid, crude ash and polysaccharide of certain composition in B-I medium were measured by air-oven method, semi-micro Kjeldahl, Soxhlet and Somogyi methods, respectively.

· After dying and fixing rotifer with a drop of Lugol reagent on the counter chamber (5×2 cm), the Rotifer was counted with Optical Microscope (Olympus Co. Ltd., ×40). After counting 3 times, the mean-value was obtained.

Results & Discussions

Cultivation and production of polysaccharide

· Cultivation of *Monilinia fructigena* and production of polysaccharide

Monilinia fructigena was cultivated in glucose medium and B-I medium for 224 hrs in 19 ℓ-bioreactor. In glucose medium, the lag phase of *Monilinia fructigena* was longer than that in B-I medium. The μ_{max} in B-I medium and glucose medium were approximately, 0.792/day and 0.816/day, respectively. Specific growth rate (μ) was faster in B-I (0.216/day) than in glucose medium (0.083/day). The maximum content of polysaccharide (0.7 g/ℓ) was also obtained in B-I medium. Santamaria et al. (1978) obtained 0.36 g/ℓ of extracellular polysaccharide with glucose medium on the flasks. Davis et al. (1964) investigated the content of extracellular polysaccharide by *Plectania occidentalis* NRRL 3137 with various carbon sources. The amouint of polysaccharide produced with glucose was 1 g/ℓ and the amounts obtained with fructose and mannose, were 0.93 g/ℓ, 0.915 g/ℓ,

respectively. It was reported that *Sclerotium rolfsii* produced 1.66 g/ℓ of extracellular polysaccharide in glucose broth with 0.017M of nitrate by Griffith & Compere (1978). Santamaria et al. (1978) observed that more extracellular polysaccharide was secreted by *Monilinia fructigena* if ascorbic acid was present in the culture. In this work, productivity of extracellular polysaccharide produced by *Monilinia fructigena* in glucose medium was 0.2~0.3 g/ℓ on the flask scale but there was no polysaccharide observed in a 19 ℓ-bioreactor. Monilinia fructigena was growing in the scaled-up bioreactor, but culture conditions to secrete polysaccharide were not suitable, so there was only little polysaccharide in glucose medium.

More extracellular polysaccharide was secreted in B-I medium which contained a large amount of different carbon source like a β-glucan. So β-glucan in the B-I medium may play a role in the production of extracellular polysaccharide as a promotor. Productivities of extracellular polysaccharide with a hydrolysate of corn cods and white fines were 0.71 g/ℓ and 1.04 g/ℓ, respectively (Compere & Griffith, 1978). In addition, *Sclerotium delphinii* and *Sclerotium glucanicum* produced a large amount of polysaccharide with sugar beet pulp, oat straw and starch (Compere & Griffith, 1978).

Photo 1 and 2 show the pictures of the mycelium of *Monilinia fructigena* in B-I medium at 73 hrs and 135 hrs, respectively. In Photo 1, it is shown that there were many thick mycelia and little polysaccharide in the culture broth. It is also shown that a lot of slim mycelia were grown out of thick ones to secrete a large amount of extracellular polysaccharide in Photo 2.

Changes of pH and dissolved oxygen (DO) in the culture and production of polysaccharide

As *Monilinia fructigena* was growing and producing organic acids, pH of the culture solution decreased to pH 3.72 in B-I medium and pH 3.6 in glucose medium. Because of the polysaccharide giving a gelatinous character to the culture, viscosity of the culture increased. The maximum content of polysaccharide occurred at the rising point from the lowest pH in the culture (Santamaria et al., 1978; Davis et al., 1965).

The degradation of the polysaccharide in the culture broth started before maximum growth was obtained. This was taken as the starting point of autolysis (Santamaria et al., 1978; Davis et al., 1965). In this experiments, the degradation of the

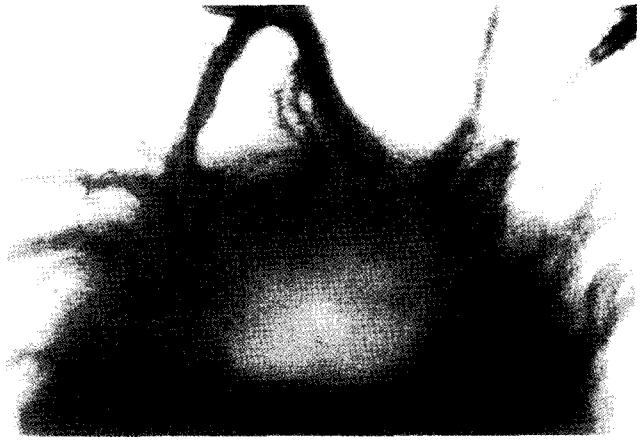

Photo 1. Mycelia of *Monilinia fructigena* after 73 hrs (×40)

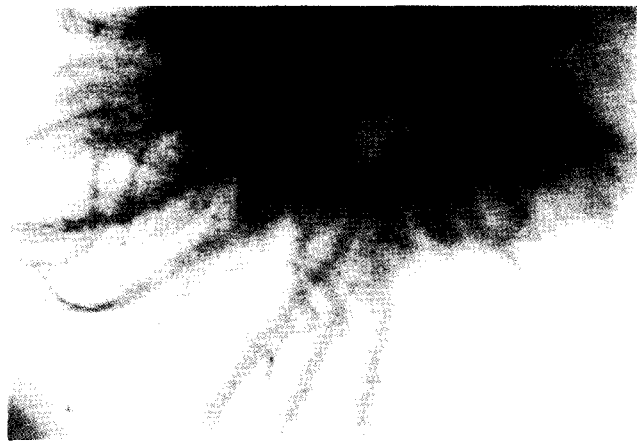

Photo 2. Mycelia of *Monilinia fructigena* after 135 hrs (×40)

secreted polysaccharide before maximum growth of *Monilinia fructigena* was convinced. Fig. 4 shows that DO in the culture broth was reducing. Despite an increasing air flow rate provided, DO of the culture solution reduced since the viscosity of the culture broth increased. After the degradation of the polysaccharide due to autolysis, viscosity of the culture decreased, so DO also increased. Fig. 3 shows growth curves of *Monilinia fructigena* in B-I medium and glucose medium. Changes of pH, DO, air flow rate and polysaccharide content in B-I medium are shown in Fig. 4.

· Viscosity of the polysaccharide suspension with B-I medium

After the mycelium suspension was filtered with 300 μm filter, the apparent viscosity of the polysaccharide suspension was measured according to the time by means of a brookfield viscometer

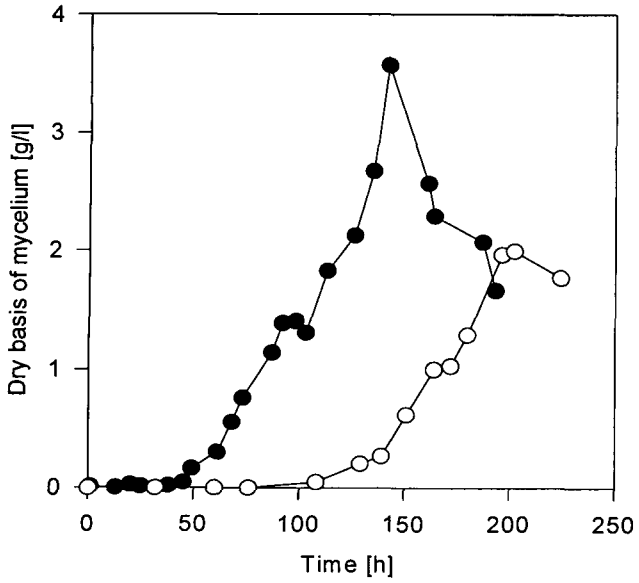

Fig. 3. Growth curves of *Monilinia fructigena* in glucose medium I (−○−) and B-I medium (−●−).

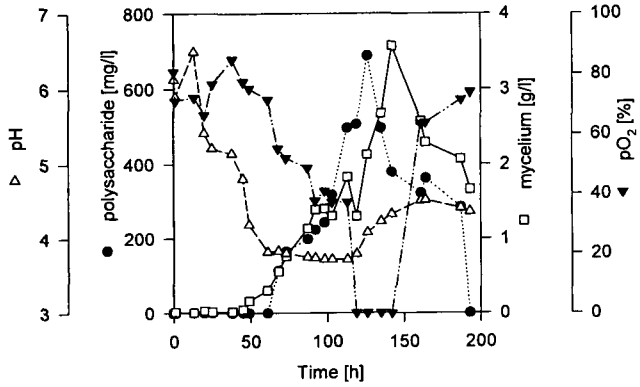

Fig. 4. Growth curve of *Monilinia fructigena* in B-I medium and changes in dry basis of polysaccharide (−●−) and mycelium (−□−), changes of pH (−△−) and pO_2 (−▼−) in culture solution.

(Brookfield Engineering Labs. Inc). As the shear rate (D) was increased, the apparent viscosity of the sample decreased. In addition, as the apparent viscosity of the polysaccharide suspension increased, the consistency Index (Kc) also increased and the flow Index (m) decreased. The polysaccharide suspension was a typical pseudoplastic fluid. Changes of the apparent viscosity of the polysaccharide suspension according to the cultivation time are shown in Fig. 5. Changes of the flow Index and consistency Index to changes of the shear rate at 73 h, 103 h, 135 h and 135 h are shown in Fig. 6.

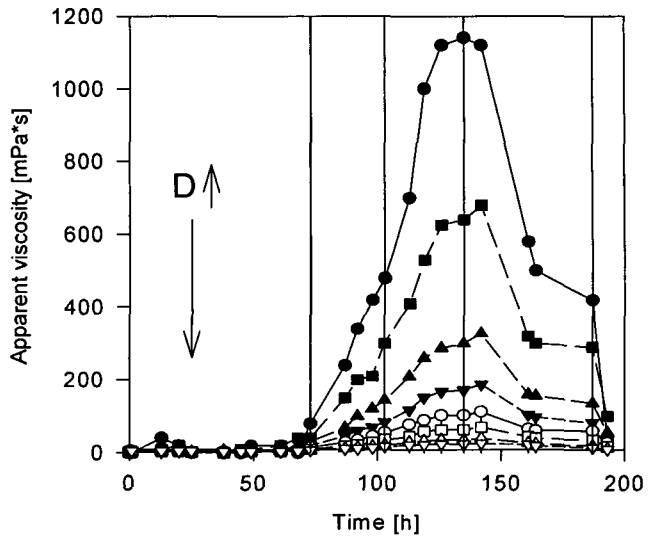

Fig. 5. Apparent viscosity of culture solution with B-I medium at several shear rate (1/sec) ; 0.066 (−●−), 0.132 (−■−), 0.33 (−▲−), 0.66 (−▼−), 1.32 (−○−), 2.64 (−□−), 6.6 (−△−) and 13.2 (−▽−).

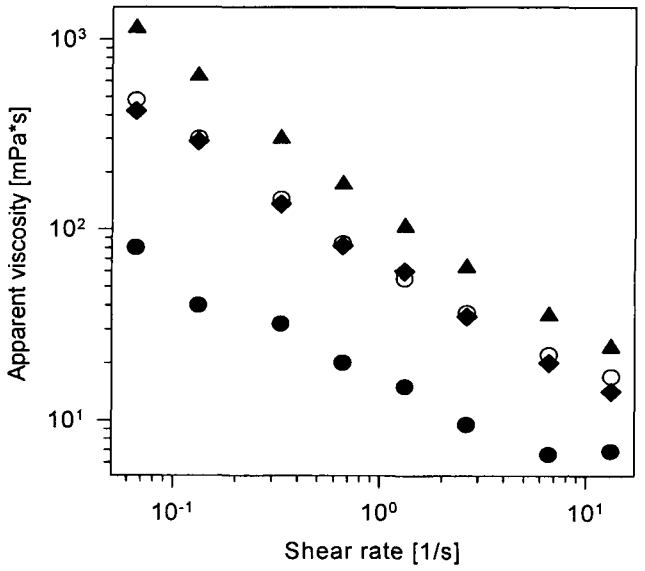

Fig. 6. Flow Index (m) and consistency Index (Kc) of culture solution with B-I medium at 73 hrs (−●−), 103 hrs (−○−), 135 hrs (−▲−) and 187 hrs (−◆−).

· Partial purification of the polysaccharide

The content of the polysaccharide was about 0.45 g/ℓ in Part A and 0.25 g/ℓ in Part B. The mycelium suspension added to the water was heated on the magnetic stirrer to separate the mycelium at 50~60℃ for 30 min. But 0.058 g/ℓ was reprecipitated

again from the used mycelium complex with the same method. If new strategies such as ultrasonication will be simultaneously attempted to precipitate the polysaccharide with the method used in this experiment, it is predictable to enhance the purification yield of the polysaccharide.

Application of the polysaccharide to rotifer

· Batch culture

The growth curves were calculated in 1 ℓ flask for 10 days. Initial concentration of rotifer was controlled to 20 cells/mℓ in each condition. The maximum concentration of rotifer was acquired after 120 hrs. In all conditions, rotifer seemed to be healthy and 2~3 eggs were born in each female. But a tremendous number of contaminants were appeared in every culture with the yeast, because the yeast feed contained a large content of protein sources and its composition was very complicated. Maximum specific growth rate of rotifer with type II was 1.095/day, higher than the others.

· Semi-continuous culture

Rotifer was cultivated in 1 ℓ flask for 30 days with the same conditions in the batch culture. After harvesting, only sea *Chlorella* sp. was supplied to rotifer as 10^6 cells per a cell. When the biomass of rotifer was greatly increased in the limited surroundings, sea *Chlorella* sp. concentrated by centrifugation was supplied to rotifer. Rotifer was harvested in logarithmic phase in the batch culture. After the number of growing rotifer in the bioreactor was harvested and set to initial number, the remained rotifer in the bioreactor were cultivated again in the fed-batch culture. Kim (1997) reported that the growth rate of rotifer supplied with *Chlorella ellipsoidea* was higher than the yeast at 26℃. But in the case of 34℃, the growth rate supplied with the yeast was much higher than *Chlorella ellipsoidea*. Therefore, if the culture temperature will be increased, the solubility of the polysaccharide will be increased, so the effect of the polysaccharide will be enhanced. Rotifer was harvested twice for 30 days. Productivity and μ_{max} in each condition are shown in Table 1. At the first harvest, μ_{max} of rotifer supplied with sea Chlorella sp. and the yeast was 1.008/day, higher than the others. But in the second harvest, μ_{max} in sea Chlorella sp. and the polysaccharide was the highest, 1.685/day. Productivity of rotifer supplied with sea *Chlorella* sp. and the polysaccharide was 38 cells/mℓ · day, whereas productivity in sea *Chlorella* sp. and the yeast was 58 cells/mℓ · day.

Table 1. Comparison of μ_{max} [day^{-1}] and Productivity [cell/mℓ · day] with 4 feeding types in semi-continuous culture

Feeding type	1st harvest		2nd harvest	
	μ_{max}	Productivity	μ_{max}	Productivity
sea *Chlorella* sp.	0.733	80	0.531	15
sea *Chlorella* sp. + β-glucan	0.734	105	1.685	28
sea *Chlorella* sp. + yeast	1.008	68	0.517	44
sea *Chlorella* sp. + β-glucan + yeast	0.495	81	1.380	22

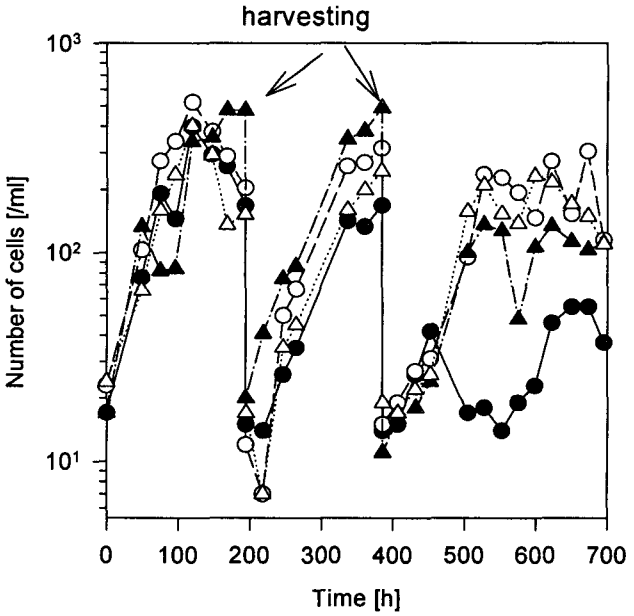

Fig. 7. Growth curves of rotifer with 4 feeding types in semi-continuous culture ; I (sea *chlorella* sp.), II (β-glucan) and III (yeast) ; I (−●−), I+II (−○−), I+III (−▲−) and I+II+III (−△−).

The growth curves of rotifer in the semi-continuous culture are shown in Fig. 7.

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