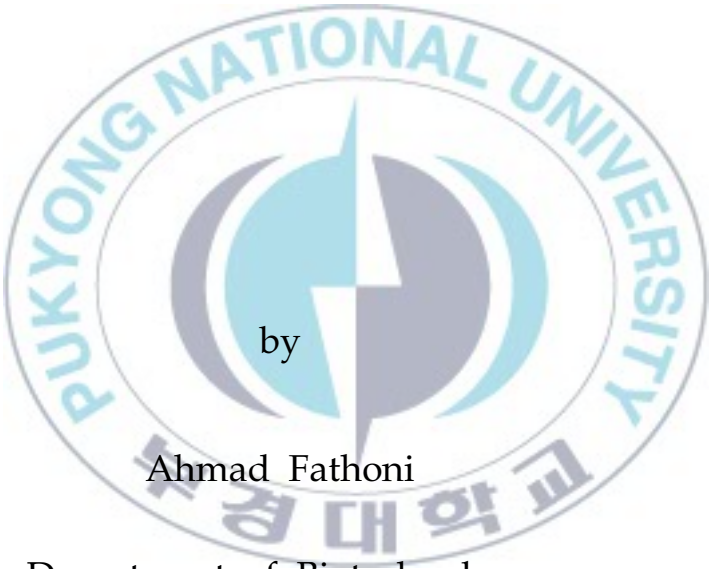
Thesis for the Degree of Master of Engineering

Biodegradation of polysaccharide in brown seaweeds, Laminaran by isolated microorganisms



by

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August, 2010

Biodegradation of polysaccharide in brown seaweeds, Laminaran by isolated microorganisms

순수 분리 균에 의한 갈조류 다당류의 분해 반응

Advisor : Prof. Joong Kyun Kim

by

Ahmad Fathoni

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Biodegradation of polysaccharide in brown seaweeds, Laminaran by isolated microorganisms

A Dissertation

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August, 2010

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Abstract

Microorganisms with the ability to produce an enzyme capable of degrading polysaccharide in brown seaweeds, particularly laminaran (β-(1,3)-glucan) were newly isolated from soil by enrichment culture contained brown seaweeds as a sole carbon source. A total six microorganisms were isolated and were studied for enzyme activities. The cellular growth of six microorganisms was monitored using spectrophotometer at OD_{375} and their ability to degrade laminaran was deduced from the increase in the amount of reducing sugars measured by dinitrosalicylic acid (DNS) method at OD_{570}. The results showed that mixed six isolated microorganisms were able to grow in the culture medium containing laminaran (5 g L^{-1}) as a sole carbon source. Compared to single isolated microorganism, mixed six isolated microorganisms also showed the higher reducing sugars amount with decreasing of pH during reaction.

Biodegradation of laminaran by mixed six isolated microorganisms was more investigated under batch conditions at 37°C and 180 rpm. The experiment was performed in 15-mL cotton-plugged tube using culture

medium containing laminaran (5 g L^{-1}), initial pH 6.8. Effects of pH changes and mixed cells composition were studied and the pattern of biodegradation product was analyzed by High Performance Liquid Chromatography (HPLC). The pH changes affected to the microorganism activity when it was adjusted with NaOH solution during reaction by decreasing of reducing sugars amount. The HPLC analysis showed that the biodegradation product formed from laminaran was not only glucose but amounts of oligosaccharides were also produced. It is indicating that the enzyme degraded linear 1,3-β-glucan laminaran in an endo splitting manner.

Glucose was detected by HPLC about 1516 mg · L^{-1} with 30.33% yield after 3 days. Based on these results, we successfully found the microorganisms which can utilize polysaccharide in brown seaweeds, laminaran. It would be important from the viewpoint of production of renewable fuels and promising for either seaweed industry or bioethanol industry.

Keyword: Biodegradation; Seaweeds; Laminaran-degrading microorganisms.

순수 분리 균에 의한 갈조류 다당류의 분해 반응

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요 약

갈조류의 구성 성분인 다당류 중에서 laminaran (β-(1,3)-glucan)를 분해 효소를 생산하는 6종의 미생물들을 단일 탄소원으로 갈조류(다시마)가 포함된 배지를 이용하여 새롭게 분리되어졌으며, 이들의 효소의 활성이 연구되어졌다. 미생물들의 세포성장은 OD_{375}에서 spectrophotometer를 사용하여 관측하였고, laminaran의 분해능은 OD_{570}. dinitrosalicylic acid (DNS) method에 의해 sugar의 환원반응실험 하였다. 실험의 결과는 혼합된 미생물들이 단일 탄소원으로서 laminaran (5 g L^{-1})을 포함하는 배지에서 성장할 수 있다는 것을 보여주었다. 또한, 단일 미생물의 배양에서와 비교하면 혼합된 6가지의 미생물들은 반응 동안에 pH 값의 감소와 함께 당 환원되는 양이 더 높게 나타난다.

혼합된 미생물에 의한 laminaran 분해 실험은 37℃와 180rpm의 회분식 배양 조건하에서 시작 pH는 6.8에서 laminaran (5 g L^{-1})을 포함하는 배지를 사용하여 15-mL cotton-plugged tube에서 수행되어졌다. pH값의 변화에 따른 혼합된 미생물들 사이에 일어나는 영향에 대하여 실험이 진행되어졌으며, 미생물에 의해서 laminaran이 분해되었을 때에 어떠한 물질이 생산이 되는지는 High performance Liquid Chromatography (HPLC)에 의해 분석되어졌다. pH의 변화에 따라 NaOH 수용액을 이용하여 초기의 값으로 맞추었을 때 미생물의 당 환원 반응의 활성에 영향을 주는 것을 알 수 있다. HPLC 분석에서는 laminaran

으로부터 분해되는 산물이 glucose 뿐만 아니라 다량의 oligosaccharide이 생산된다는 것을 보여줌으로서 미생물들이 endo splitting manner에서 선형의 1,3-β-glucan laminaran를 분해하는 효소가 있다는 것을 알 수 있다.

미생물들의 배양 3일 후에 glucose는 1516 mg · L^{-1} 생산되어졌으며, 이때의 수율은 30.33%이다. 이 결과로 갈조류의 구성 성분인 다당류 중에서 laminaran를 분해 효소를 생산해내는 미생물들을 발견하였음을 알 수 있었으며, 이러한 해조류를 이용하여 당을 생산해내는 미생물들을 이용한다면, bioenergy 사업에서 renewable 연료 생산에서 원료로서 사용될 것이라고 기대 된다.

I. INTRODUCTION

1. Characteristics of brown algae

The term algae refer to a large and diverse assembly of eukaryotic organisms that contain chlorophyll and can carry out oxygenic photosynthesis. Most algae are microscopic and unicellular, but in this assembly we also find big multicellular organisms, the seaweeds. Brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta) are the common groups of macro algae. They are typically found along coastlines down to 50 meters, and live attached to the bottom by specialized structures called holdfasts. This sea environment is rather stable in temperature, humidity and salinity. Globally, the present utilization of seaweeds may be divided into the consumption of algae as food in the Orient, and the industrial use of phycocolloids throughout the world.

Brown algae are divided in 9 orders, 265 genera and more than 1500 species. They absorb medium wavelength green light, which enables them to live even at 30-50 m depths, but the majority live in the intertidal belt and upper sublittoral zone. Brown algae prefer cooler water temperatures than red and green algae. Immersed in water, the seaweeds have no need for

internal transport of nutrients or water (Gao and McKineley, 1993). Thus, brown seaweeds have a high potential for biomass production and CO_2 fixation, and may be an attractive alternative source for energy and chemicals (Svein, 2000). Laminaran used on this study was extracted from *Laminaria digitata* (fig.1)

Fig.1. *Laminaria digitata;* Dark brown, to 2 m in length; with a claw-like holdfast, a smooth, flexible stipe, and a laminate blade to 1.5 m long split into finger-like segments.

2. Chemical composition of seaweeds

The chemical composition of brown algae varies considerably between species, throughout the year and between habitats. Brown seaweeds exposed to seasonal changes usually accumulate mannitol and laminaran in the light season (spring to autumn), and consume these carbohydrates during growth in the dark season (Haug and Jensen, 1954).

The table below shows the chemical composition of selected, representative seaweeds, some of which are currently used for food or have been used as food in the past. All figures, except for water (as percentage), are given as grams per 100 grams of dry matter. When no data are available "nd" is inserted.

In regard to the amount of protein, the convention is to convert the total nitrogen to protein by multiplying by 6.25. This should be treated with some caution as, for example, the amount of free nitrate will affect the total nitrogen level. Free nitrates are found in varying amounts in red and brown algae.

Table 1. Chemical composition of seaweeds.

(Gayral, P. & Cosson, J. 1973, Haug, A. & Jensen , A. 1954, Jensen, A. 1956, Jensen, A. 1956)

Composition	*Laminaria digitata*	*Ascophyllum nodosum*	*Palmaria palmata*	*Ulva species*
Type	Brown	Brown	Red	Green
Water (%)	73-90	70-85	79-88	78
Ash	73-90	15-25	15-30	13-22
Total carbohydrate	-	-	-	42-46
Alginic acid	20-45	15-30	0	0
Xylans	0	0	29-45	0
Laminaran	0-18	0-10	0	0
Mannitol	4-16	5-10	0	0
Fucoidan	2-4	4-10	0	0
Floridoside	0	0	2-20	0
Other carbohydrate	1-2	c.10	nd	nd
Protein	8-15	5-10	8-25	15-25
Fat	1-2	2-7	0.3-0.8	0.6-0.7
Tannins	c. 1	2-10	nd	nd
Potassium	1.3-3.8	2-3	7-9	0.7
Sodium	0.9-2.2	3-4	2.0-2.5	3.3
Magnesium	0.5-0.8	0.5-0.9	0.4-0.5	nd
Iodine	0.3-1.1	0.01-0.1	0.01-0.1	nd

3. Storage carbohydrates from brown seaweeds; Laminaran

In brown seaweeds, alginate is the main structural compound (Kloareg and Quatrano, 1988), while mannitol and laminaran are common storage materials. Thus, the absence of lignin and the low content of cellulose in brown algae should make them a simpler material for bioconversion than land plants. Seaweeds rich in carbohydrates and are most favourable for biological degradation with a low content of ash and water. However polyphenols (Morand et al., 1991) and salt (Ghosh, 1988 and Moen, 1997) in the algae may reduce the biodegradability.

The polysaccharides laminarin, the storage glucan found in most algae and phytoplankton (Meeuse, 1962 and Painter, 1983), is one of the most abundant carbohydrates in the marine ecosystem. It consists of liner, mannitol- or glucose-ended chains of β-(1→3)-linked glucose residues, with occasional β-(1→6)-linked branches (Maeda and Nisizawa, 1968; Elyakova and Zvyagintseva, 1974; Usui et al., 1979; Read and Currie, 1996) (fig. 2). The main chain length and the overall degree of polymerization (DP) of laminarin range from 7 to 19 and 16 to 31, respectively, indicating a structural polydispersity, from essentially linear β-(1→3)-glucans to branched β-(1→3), β-(1→

6)-glucans with an average of three ramifications per molecule. Solubility in cold water depends on the branching characteristics, with increasing linearity resulting in a greater ability to form intermolecular hydrogen bonds and insoluble aggregates (Bull and Chesters, 1966). It is a soluble β-1,3-D-glucose polymer with some branching at positions C-2 and C-6, and is also known as laminaran or leucosin. The size typically ranges from 20-30 glucose residues, and some chains are terminated by mannitol end-groups (Meeuse, 1962; Painter, 1993 and Read et al., 1996)

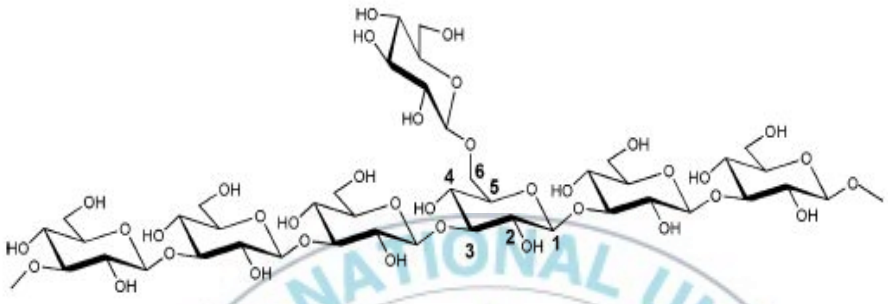

Fig 2. Chemical structure of 1,3-β-D-glucan with 1,6-linked branches of glucopyranosyl unit.

There are two types of enzyme involved in the hydrolysis of polysaccharides; an exo and an endo-type enzyme. The exo-type enzyme (E.C 3.2.1.58) hydrolyzes the non reducing ends of β-1,3-D-glucan to form β-D-glucose, whereas the endo-type enzyme (EC 3.2.1.39) randomly hydrolyzes the β-1,3-D-glucosidic linkage in β-D-glucans and another endo-type enzyme (EC. 3.2.1.6) also randomly hydrolyzes the β-1,3- and β-1,4-glucosidic linkages in β-D-glucans (Miyanishi et al., 2003).

To date, β-(1→3)-glucanase has been isolated from various sources such as plant (Ketoke et al., 1997 and Akiyama et al., 1998), bacteria (Horikoshi, 1997 and Kanazawa) and yeast (Molina et al. and Esteban et al). β-(1→3)-glucanase that can hydrolyze laminaran to glucose monomer where it is a good substrate for fermentation process. Mannitol, on the other hand, is not readily fermented. It is initially oxidized to fructose by the enzyme mannitol dehydrogenase (Litkenhous, 1967).

4. Seaweed used as human food

Seaweeds have been used as a human food since ancient times, particularly in China, Japan and Korea. Seaweeds have also been consumed by the coastal populations of many

countries, sometimes as part of a subsistent living, or as a regular ingredient of salad-type preparations, the latter especially in Hawaii and the warmer countries of South East Asia such as Indonesia, Malaysia, Philippines and Thailand (http://www.fao.org/docrep/004/y3550e/Y3550E04.htm).

Recently, the Korean is one of the biggest consumers of seaweed as food. New method have been developed for the handling, treatment and sorting of the seaweeds to meet marketing requirements. The strict selection and subsequent grading for an accepted product resulted in excessive "waste material." In order to obtain a maximum benefit from the available resource, methods have been developed to convert this low-grade, unmarketable seaweed into more useful compound. Seaweed may have a high content of degradable carbohydrates, making them a potential substrate for the production of bioethanol (Rotmann, 1987).

5. Production of ethanol from seaweeds

Nowadays almost all bioethanol, i.e. ethanol produced from renewable resources, is produced in Brazil, USA and Canada. The majority of the bioethanol is used in the transport sector as an oxygenated fuel additive. The raw material used is

sugar-based or starch-based (Wheals, 1999). Over the last few decades, ethanol production from renewable resources has been of interest as an alternative fuel.

There are few reports about any other research done on ethanol production from seaweeds. Most of the work concerning bioconversion of seaweed has been related to methane gas production. The reason may be the complex composition of seaweeds, containing several different carbohydrates. Finding a microorganism that can ferment all the different carbohydrates to ethanol is not very likely. Besides, alginate, as a major component in brown algae, may not be fermented to ethanol since the redox balance in the glycolytic oxidation of uronic acids to pyruvate is maintained. Hence, no excess electrons are available for the reduction of pyruvate to ethanol. On the other hand, Laminaran and mannitol may be converted to ethanol, and these sugars can also easily be extracted from milled seaweed (Percival and McDowell, 1967).

In this study, we were interested in isolating useful microorganism that can utilize polysaccharides found in brown seaweeds. A microorganism capable of degrading the polysaccharides found in brown seaweeds (laminaran) will be important from the viewpoint of production of renewable fuels and promising for either seaweed industry or bioethanol industry.

Ⅱ. MATERIALS AND METHODS

1. Materials

At the beginning, brown algae (seaweeds) were used for screening the laminaran degrading bacteria. The brown seaweeds were obtained from the market and standard laminaran from *L. digitata* was purchased from Sigma-Aldrich, Co., Korea. Stock solution of laminaran (10% w/v) was made by dissolving 1 g of laminaran into 10 mL of distilled water. It was then autoclaved at 121℃ during 15 min for sterilization.

The release of biodegradation product was measured as a reducing sugar by the dinitrosalicylic acid (DNS) method. The DNS reagent was made by dissolving 0.25 g of 3,5-dinitrosalysilic acid and 75 g potassium sodium tartrate (Rochelle salt) in 50 mL of 2M-NaOH and diluting to 250 mL with distilled water (Chaplin, 1986 and Miller, 1959)

2. Microorganisms and culture conditions

The potential microorganisms with the ability to degrade polysaccharide in brown seaweeds, Laminaran were isolated from soil samples which taken from land area located nearby laboratory in the early of July. Microorganisms were screened from soil using liquid medium (pH 6.8) contained sterile tap water, 0.1 g L^{-1} of NH_4Cl and brown seaweeds (1 mm^2 in size) as a sole carbon source.

Screening of useful microorganisms was conducted in a 250-mL flask, which was incubated at 37℃ and 180 rpm for 8 days. After incubation, samples were streaked onto an agar medium (pH 6.8) contained 8 g L^{-1} of Nutrient broth with 1.5% (w/v) of agar. Streaked agar plates were incubated at 37℃ and for 3 days. Mixed colonies formed on agar were purified by repeated streaking onto new agar medium. Each pure culture was maintained on the agar plate and stored at 4℃. It then periodically transferred onto new agar medium every 4 weeks. In order to maintain the microbial activity on degradation of laminaran, each pure culture was also grown both in liquid medium and on agar medium contained 1 g L^{-1} of laminaran then stored at 4℃ and -20℃ until use.

3. Inhibitory effects of isolated microorganisms

The isolated microorganisms were tested for the inhibitory effect or potential of bacterial antagonists against each others. This experiment was done by using a perpendicular streak technique as described by Litkenhous and Liu (1967), a streak of 20 mm of potential antagonist strain was made with a sterile cotton swab across a plate of laminaran medium containing 1.5% of agar.

The plates were incubated at 37℃ for 5 days to allow the production of antagonic substance and then were checked of any growth inhibition of each isolate.

4. Effects of vitamin and mineral on microbial growth

In order to investigate the effects of vitamin and mineral on cell growth against control, vitamin 0.05% (v/v) and mineral solutions 0.05% (v/v) were added into the laminaran medium. After autoclaving, 0.5 mL of vitamin solution and 0.5 mL of mineral solutions, which have been sterilized by filtering through the 0.2 μm filter papers, were added into the laminaran medium. Cultivation was carried out in a 100-mL flask at 37℃ and 180 rpm, with 5% (v/v) of inoculum size. Cellular growth was measured periodically as absorbance or optical density at 375 nm by using a VIS/UV spectrophotometer OPRON-3000.

5. Biodegradation of laminaran by isolated microorganisms

The ability of isolated microorganisms to degrade polysaccharide in brown seaweeds laminaran was investigated. The biodegradation of laminaran was carried out in batch culture of 15-mL cotton plugged tube with an initial working volume of 8 mL using a laminaran medium, which contained (per L): 0.1 g of $MgSO_4$, 0.1 g of NaCl, 0.1 g of $CaCl_2$, 2 g of $(NH_4)_2SO_4$, and 0.5 g of KH_2PO_4 and 5 g of laminaran. Before running the experiment, cells were adapted for 5 days in the medium contained laminaran (1 g L^{-1}) at 37℃ and 180 rpm and harvested by centrifuging at 7,000 rpm for 10 min. With 0.2% (w/v) of inoculum size, cells were inoculated into the liquid medium contained laminaran (5 g L^{-1}). and incubated in shaking incubator at 37℃ and 180 rpm.

The release of biodegradation product from laminaran was determined as reducing sugar by the DNS method. And the pattern of biodegradation product was analysed by High Performance Liquid Chromatography (HPLC).

6. Effects of pH changes and mixed cells composition on microbial activity

The effects of pH changes on the biodegradation of laminaran by isolated microorganisms was investigated by adjusting pH solution to the initial state (pH 6.8) with adding alkaline solution. Samples were added with NaOH (1N) solution at the sampling time.

In the study of the effects of mixed cells composition on degradation of laminaran, we applied different composition of mixed cells into the laminaran medium. In this study, we used mixed cells (FS1 to FS6) and mixed cells (FS2 and FS4). The effects of pH changes and mixed cells composition were observed by measuring the reducing sugar using dinitrosalicylic acid (DNS) method.

7. Methods of analysis

7.1. Determination of cell growth

Cell growth of isolates was determined by measuring the optical density using a VIS/UV spectrophotometer OPRON-3000 at the fixed wavelength at 375 nm.

7.2. Determination of enzyme activity

The enzyme activity of isolated microorganisms on degradation of laminaran was deduced from the increase in the amount of reducing sugars measured by dinitrosalicylic acid (DNS) method. Culture sample (150 μL) were taken and centrifuged using 1.5 ml eppendorf tube at 7,000 rpm for 5 min. The supernatant (100 μL) was then pipetted and added into 1 mL dinitrosalicylic acid reagent and mixed well. The mixed solution was heated at 90-95℃ for 10 min. After rapid cooling to room temperature, reducing sugar was determined at OD 570 nm (Chaplin, 1986 and Miller, 1959) by using a VIS/UV spectrophotometer OPRON-3000.

7.3. High Performance Liquid Chromatography (HPLC) analysis

Pattern of biodegradation product formed from laminaran by isolated microorganisms was examined using HPLC. Culture samples (1 mL) were centrifuged at 7,000 rpm for 10 min. to remove bacteria. The supernatant was filtered with 0.2 μm pore size and was analyzed by using HPLC. The HPLC system was equipped with refractive index (RID) detector. The HPLC column used was Sugar-Pak I 300 mm x 6.5 mmID. The mobile phase used was H_2O, flow rate was set at 0.5 mL/min. The column and detector temperatures were set at 80℃ and 35℃, respectively. The degraded products were detected by HPLC with glucose as the standard.

Ⅲ. RESULTS AND DISCUSSION

1. Screening of useful microorganisms

Microorganisms with the ability to produce an enzyme which is able to degrade polysaccharide from brown seaweeds, Laminaran were screened from soil with enrichment culture containing brown seaweeds as a sole carbon source.

After incubation at 37℃ for 3 days, colonies appeared on agar plates and dominant colonies were selected as microorganisms with the ability to metabolize polysaccharide from brown seaweeds. The colonies of screened microorganisms were purified by repeated streaking on nutrient agar and incubated at 37℃. Six strains were successfully isolated and their characteristics in liquid medium were observed by direct microscope under 1,000 magnifications (Table 2). The six strains were named as FS1, FS2, FS3, FS4, FS5 and FS6 respectively. They were tested for the ability to metabolize polysaccharide from brown seaweeds especially laminaran. In order to maintain their activity, six strains were periodically transferred onto the fresh agar medium contained laminaran (1 g L^{-1}) every 4 weeks and were stored at .4℃.

The six strains formed colonies on agar medium containing 1 g L^{-1} of laminaran and 1.5% of agar Among six strains, FS3 formed colony weakest than others (fig. 3).

Table 2. Microscopic observation of screened microorganisms under 1,000 magnification.

Isolate	Characteristics
FS1	Spore forming bacteria, short rod shaped, 1-2 μm (L), 1.0-1.5 μm (W), catalase positive and gram positive.
FS2	Short rod shaped bacteria, 1-2 μm (L), 0.5-1.5 μm (W), catalase positive and gram negative.
FS3	Thin rod shaped bacteria, 2-4 μm (L), 0.4-0.7 μm (W), catalase positive, and gram negative.
FS4	Irregular shaped bacteria, cluster, 0.5-1.5 μm (L), 0.5-1.0 μm (W), catalase positive and gram positive.
FS5	Spore forming bacteria, rod shaped bacterium, 3-5 μm (L), 1-1.5 μm (W), catalase negative and gram positive.
FS6	Spore forming bacteria, short rod shaped bacterium, 1-2 μm (L), 0.5-1.0 μm (W), catalase positive and gram positive.

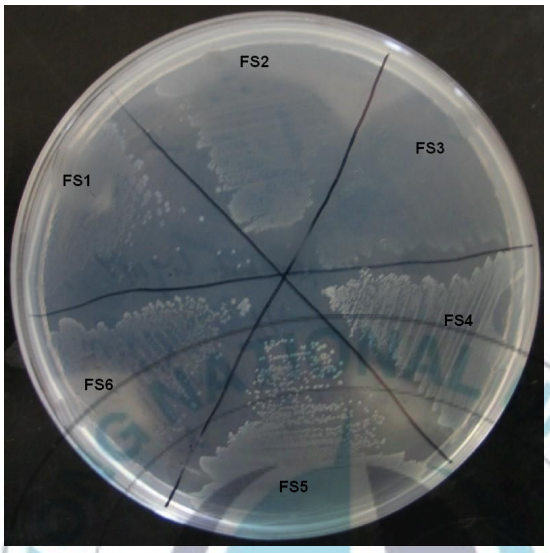

Fig. 3. Colony forming of six isolated microorganisms on agar medium contained laminaran (1 g L^{-1}).

2. Inhibitory effects of isolated microorganisms

According to the previous study by A.M. Alippi et al. (2006), some bacteria strains showed antagonistic activity against others. It could be related to the production of bacteriocins or bacteriocin-like compound which can inhibit to the cell growth.

Inhibitory effects study allow to investigate the possibility for using them together in reaction. In this study, the inhibitory effect of each isolate has been tested using perpendicular streak technique as described by Litkenhous and Liu (1967). Each isolate was streaked with a sterile swab across a plate and the plate was incubated at 37℃. After incubation, the plates were checked for the inhibitory effect. The result showed that among six isolated microorganisms, there was no growth inhibition showed by each isolate. It can be confirmed that each isolate could grow on agar medium as shown in fig 4.

These results also describe the microbial interaction among the isolated microorganisms in the culture medium and allow us to apply them together on biodegradation of laminaran.

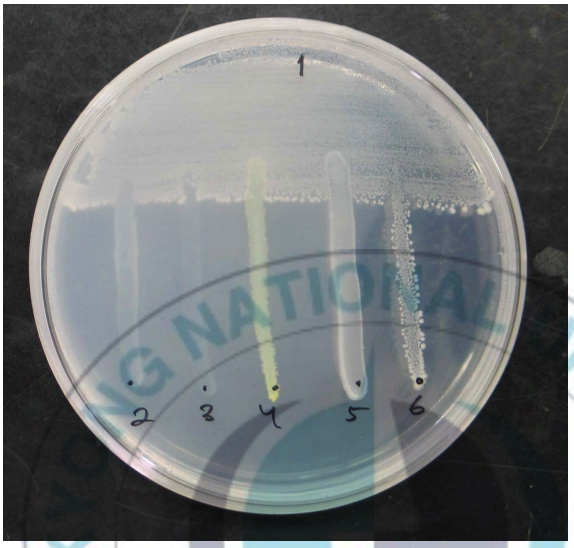

Fig. 4. Inhibitory effect of FS1 against other species on agar medium. Other data are not shown (same results).

3. Effect of vitamin and mineral on microbial growth

In microbial growth, cells must active the metabolic pathway for amino acids and vitamins to begin active growth. The effects of vitamin and mineral on the cellular growth of six isolated microorganisms were studied with adding and without adding vitamin and mineral into the culture medium containing laminaran (10 g L^{-1}).

As shown in fig. 5, the results showed that the cellular growth of six isolated microorganisms was influenced by the presence of vitamin and mineral in the culture medium. Cells reached a stationary phase within 2 d, which was faster than observed in the absence of vitamin and mineral solutions. The factors that cause cells to enter stationary phase are related to changes in environment, typically caused by high cell density. It means the shorter time required by cells to enter the stationary phase the higher cell density in the culture medium.

Specific growth rates (μ) with the presence and the absence of vitamin and mineral solutions were calculated to be 0.23 and 0.14 h^{-1}, respectively. It means that vitamin and mineral solutions were helpful for bacteria to grow and metabolize laminaran in the culture medium.

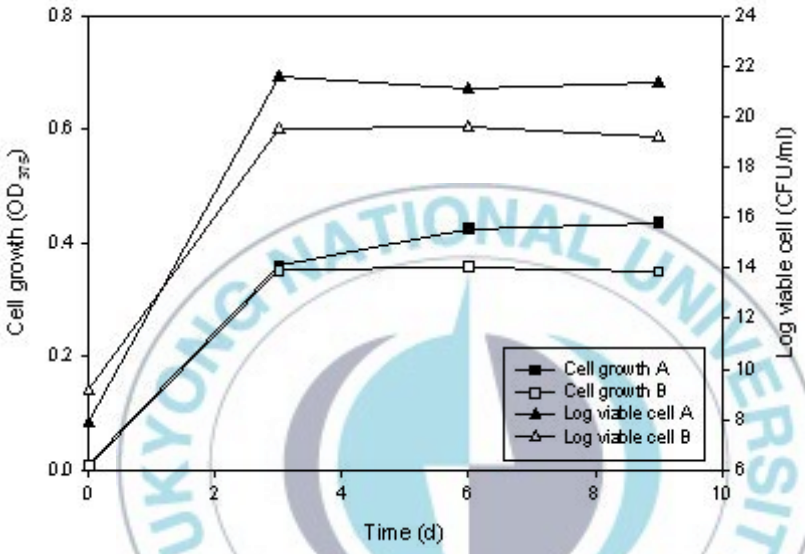

Fig. 5. Effect of vitamin and mineral on cell growth. Cells were cultivated in the medium with presence (A) and absence (B) of 0.05% vitamin and 0.05% mineral solutions.

4. Biodegradation of laminaran by isolated microorganisms

In the initial study, biodegradation of laminaran was investigated by applying mixed six isolated microorganisms against single cell. This experiment was performed in 15-mL cotton-plugged tube using liquid medium contained laminaran of 5 g L^{-1} and data were taken during 9 days experiment. The cellular growth and reducing sugar amounts produced by single cell against mixed cells on degradation of laminaran are shown in fig. 6 and fig. 7, respectively. Compared to single cell, mixed cells grew better in liquid medium contained laminaran and also produced higher amounts of reducing sugar, whereas the single cell showed very low degrading activity.

These results indicate that laminaran could be easier degraded by mixed cells to its simpler sugar than single cell. Based on this results, the optimum activity of mixed cells on degradation of laminaran needs to be more investigated.

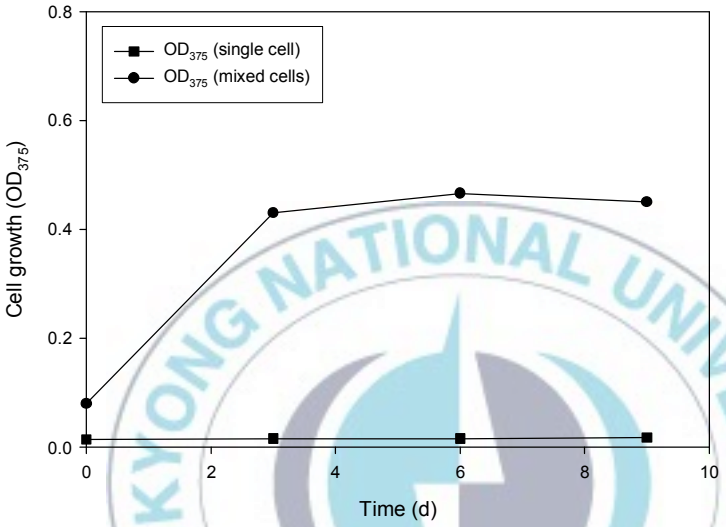

Fig. 6. Cellular growth (OD_{375}) of mixed cells (-●-) against single cell (-■-) in liquid medium contained laminaran (5 g L^{-1}) with 6 fold. diluted sample.

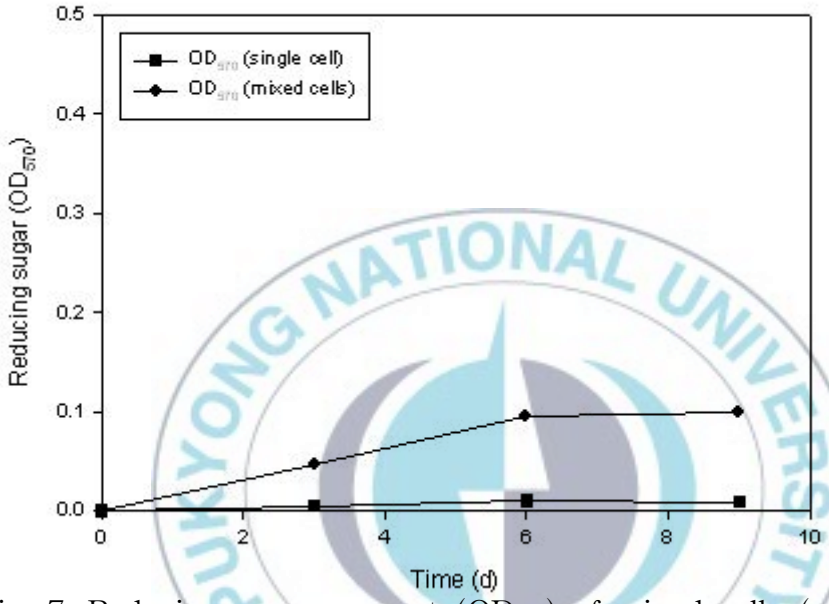

Fig. 7. Reducing sugar amount (OD_{570}) of mixed cells (-●-) against single cell (-■-) in liquid medium contained laminaran (5 g L^{-1}) with 3 fold. diluted sample.

5. Profile of pH on biodegradation of laminaran by mixed isolated microorganisms

During reaction, pH values of culture medium were measured by pH meter. The profile of pH on biodegradation of laminaran during reaction is shown in fig. 9. The pH decreased to the range of 4.5-5.0 from the initial pH of 6.8. The pH optima of β-(1,3)-glucanase from oats, barley, potato, hyacinth, soya bean, actinomycetes and seaweeds are close to our values (Dillon & O'Colla, 1951; Peat et al. 1952; Duncan et al. 1956; Preece et al. 1960).

It has been reported that the acidic pH resulting from degradation of laminaran by mixed isolated microorganisms is probably caused by the production of short chain fatty acid (SCFA) (Deville, 2007). Short chain fatty acid such as acetic acid is miscible with water and dissociate to form reasonably strong acids, so that it has significant effects on the pH of a solution. Short chain fatty acids which produced by isolated microorganisms may be a mechanism of intestinal resistance to their new environment in liquid medium containing laminaran.

Some water-soluble fibers, such as resistant starch from potatoes and undigested oligosaccharides, are metabolized to short chain fatty acids (SCFA; acetic acid, propionic acid and butyric acid) and lactic acid by colonic bacteria (Cherbut, Michel, & Lecannu, 2003). Laminaran from *Laminaria digitata* is water soluble polysaccharide that could be also metabolized to short chain fatty acids. Other studies showed the production of fatty acids from laminaran by various bacteria. Short chain fatty acids (acetic acid, propionic acid and butyric acid) and lactic acid were produced from degradation of *Laminaria digitata* by human fecal bacteria (Deville, 2007; Kuda, 2005; Rochet, 1997 and Kuda, 2009)

In a study by Levison (1973), the production of short chain fatty acids (acetic acid, butyric acid) and lactic acid inhibited the growth of bacteria. In this study, on the other hand, the production of short chain fatty acids cause the acidic pH which helped the isolated microorganism degrade polysaccharides from seaweeds, especially laminaran.

More investigation concerning the production of short chain fatty acids (SCFA) on biodegradation of laminaran by mixed isolated microorganism are needed. Short chain fatty acids can be analysed by using gas-liquid chromatography (GLC).

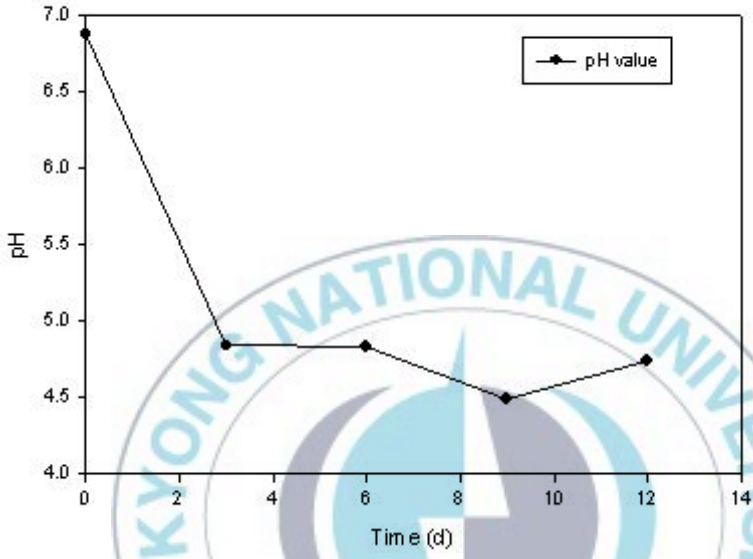

Fig. 8. Profile of pH during degradation of laminaran by isolated microorganisms.

7. Effects of pH changes and mixed cells composition on microbial activity

7.1. Effects of pH changes

In the initial study showed that pH of culture medium decreased to range of 4.5-5.0 during reaction. In more study about pH on the reaction, we investigated the effect of pH changes on the enzyme (β-(1,3)-glucanases) activity from isolated microorganisms. To carry out this experiment, pH value was adjusted to the initial pH by adding alkaline solution using NaOH (1 N) at the sampling time during experiment. Effect of pH changes on both microbial growth in the culture medium and their activity to degrade laminaran are depicted in fig. 10 and fig. 11, respectively. The results showed that the growth of isolated microorganism was lower when the pH was adjusted. And the reducing sugar amount also decreased when pH was adjusted. Briefly, the pH changes affected to the cell growth and reduce the ability of β-(1,3)-glucanases activity from isolated microorganisms to degrade laminaran when the pH was adjusted.

The results indicate that β-(1,3)-glucanases are sensitive to pH changes in their environment. In the previous study, Chesters and Bull (1962) reported that this enzyme was sensitive to pH changes and inactivated at extreme values. β-(1,3)-glucanases either endo or exo-type were most active at lower pH (4-6).

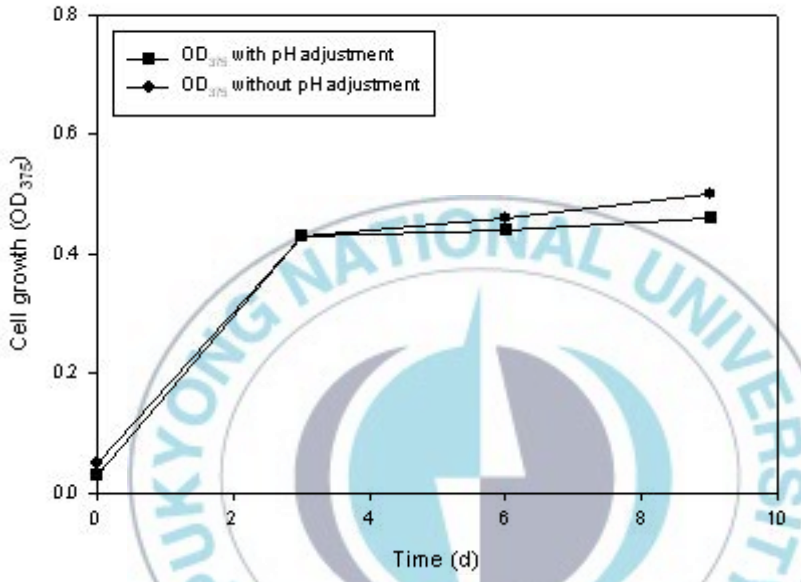

Fig. 9. Cellular growth (OD_{375}) of isolated microorganism in the culture medium contained laminaran (10 g L^{-1}). without pH adjustment (-●-) and with pH adjustment (-■-) with 6-fold diluted samples.

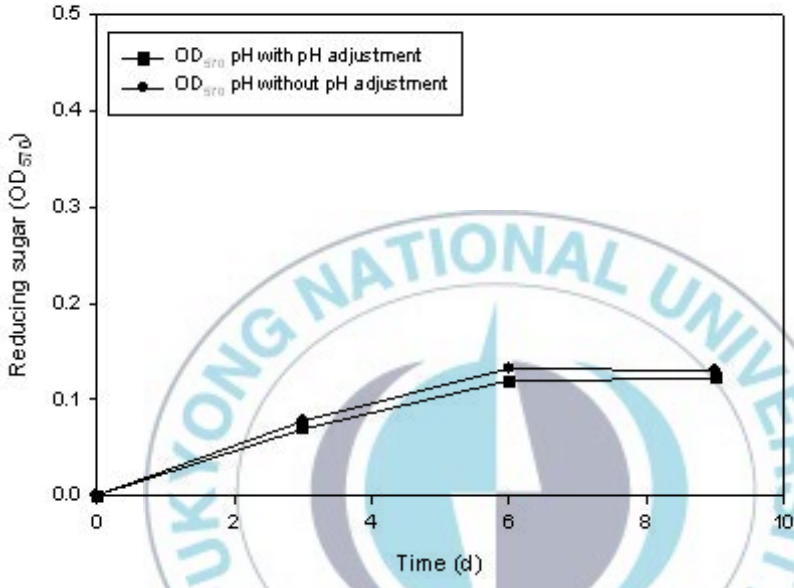

Fig. 10. Reducing sugar amounts in the culture medium without pH adjustment (-●-) and with pH adjustment (-■-) with 3-fold diluted samples.

According to these results, pH could become a crucial factor on biodegradation of laminaran. However, more study about the role of pH on biodegradation of laminaran are needed.

7.2. Effects of mixed cells composition

In the previous results, the activity of single cell against mixed cells on degradation of laminaran has been investigated. We obtained that the mixed cells grew better in liquid medium containing laminaran and produced higher amounts of reducing sugar than single cell. Under microscopic observation at 1000 magnifications, there were two strains (FS2 and FS4) that dominantly grew in culture medium among six isolated microorganisms. Hence, the effect of mixed cells composition was done by investigating on two strains (FS2 and FS4) against mixed six strains. The results are shown in fig. 12 and fig. 13.

The results show that the cellular growth both mixed six and two strains significantly increased within 3 days and entered to stationary phase after that time with the specific growth (μ) of 0.06 and 0.07 h^{-1}, respectively. Compared to mixed six strains, the production of reducing sugar by mixed two strains was higher. It is suspected that between two strains there are mutual interaction to degrade polysaccharide in brown seaweeds, laminaran.

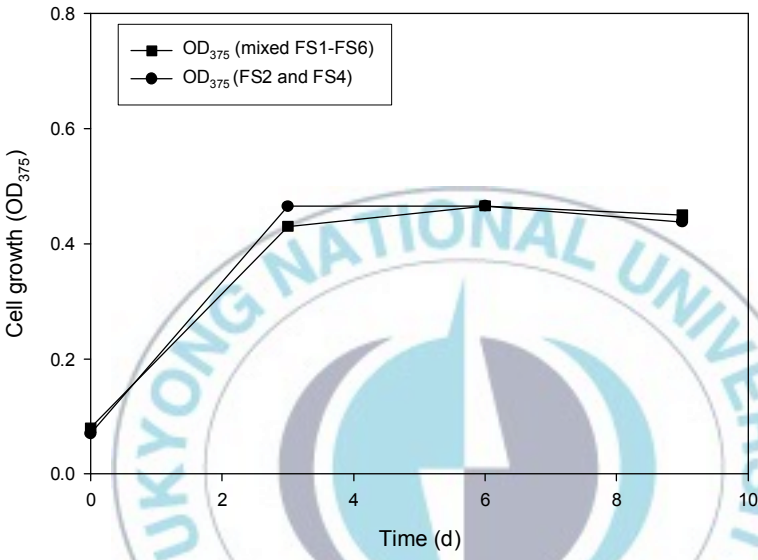

Fig. 11. Cellular growth (OD_{375}) of mixed six cells (FS1-FS6) (-■-) against mixed two cells (FS2 and FS4) (-●-) in liquid medium contained laminaran (5 g L^{-1}) with 6-fold diluted samples.

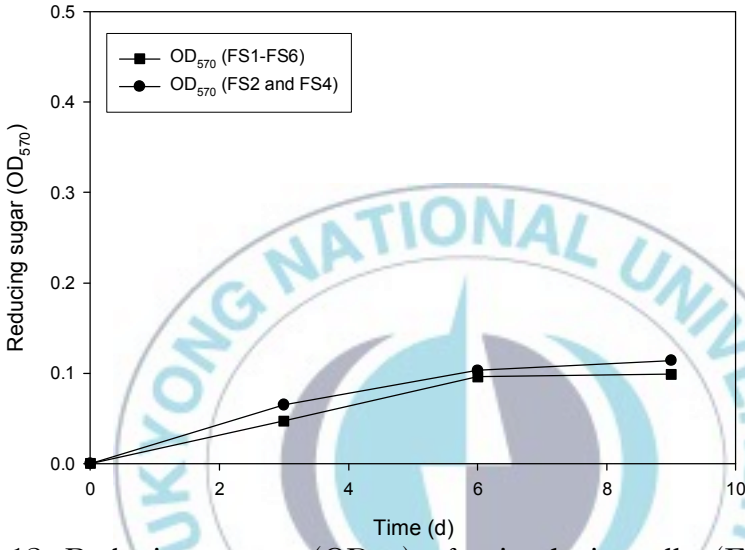

Fig. 12. Reducing sugar (OD_{570}) of mixed six cells (FS1-FS6) (-■-) against mixed two cells (FS2 and FS4) (-●-) in liquid medium contained laminaran (5 g L^{-1}) with 3-fold diluted samples.

Based on these results, the enzyme activity from isolated microorganisms was influenced by the composition of mixed cells due to their mutual interaction in the culture medium to degrade laminaran. However, the further study about their interaction and mixed cells composition to obtain the optimum results are needed.

5. Pattern of biodegradation product by mixed six isolated microorganisms

The pattern of biodegradation product by mixed six isolated microorganisms was analyzed by using HPLC. The results showed that the biodegradation product formed from laminaran was not only glocuse but amounts of oligosaccharides (the larger sugar than glucose) were also produced (fig. 8).

The similar results were shown in other studies. Product of degradation of laminaran by endo.-β-(1,3)-glucanases from *Haliotis tuberculata and Bacillus clausii* NM-1 were glucose, laminaribiose and laminaritriose (V.Lepagnol-Descamps et al., 1998; Miyanishi et al., 2002), by β-(1,3)-glucanases from *Trichoderma harzianum* were glucose, gentibiose and laminaribiose (E.C. Giese, 2006). Endo.-β-(1,3)-glucanases from

Perna viridis produced glucose and di-,tri-,tetra-saccharides (Zakharenko et al. 2008) and .Endo.-β-(1,3)-glucanases from Marine mollusk *Littorina kurila* produced Glucose, laminariobiose, other small oligosaccharides (M.S. Pesentseva et al. 2008).

This result confirms that mixed isolated microorganisms produced an enzyme β-(1,3)-glucan that degrade polysaccharide from brown seaweeds, laminaran. And by using the HPLC analysis we can investigate the type of enzyme produced by microorganisms wheather it is endo-enzyme or exo-enzyme. The production of glucose and amounts of oligosaccharides with different sizes indicate that β-(1,3)-glucanases from the mixed isolated microorganisms degraded linear β-(1,3)-glucan laminaran in an endo-splitting manner.

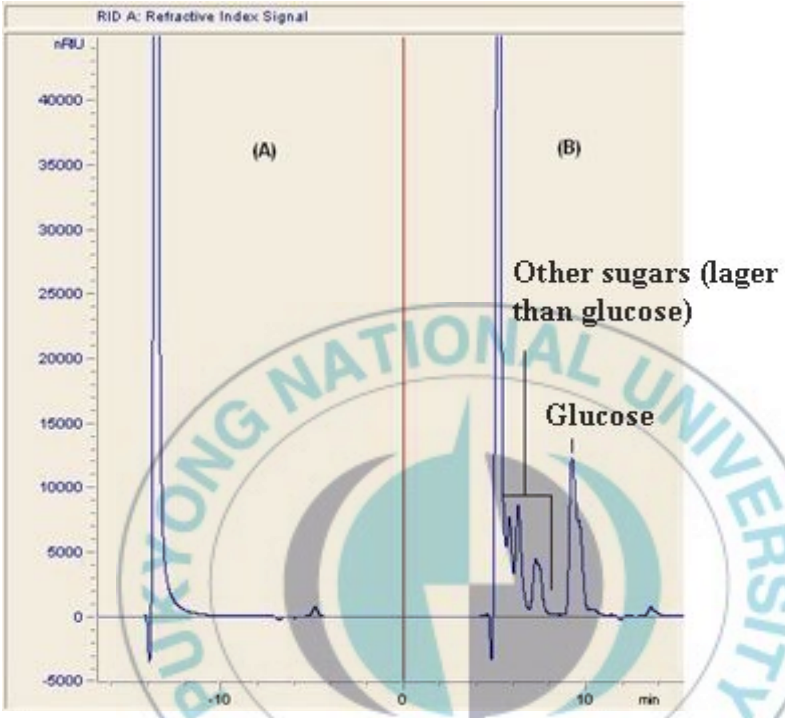

Fig. 13. HPLC analysis of a standard laminaran (a) and product by mix isolated microorganisms (b).

Ⅳ. Conclusion

In order to utilize the polysaccharide in brown seaweeds, especially laminaran, six strains of bacteria were successfully isolated from soil. These bacteria were named as FS1, FS2, FS3, FS4, FS5 and FS6 respectively. In the first study on microbial activity showed that mixed six isolated microorganisms were able to grow better in the liquid medium contained laminaran (5 g L^{-1}) than single cell. The cellular growth of six isolated microorganisms was influenced by the presence of vitamin and mineral solutions in the medium.

The acidic pH resulting during reaction indicated that pH could be a crucial factor on biodegradation of laminaran. Reducing sugar amount produced by mixed isolated microorganisms was influenced by pH changes and mixed cells composition. From the results were obtained that mix isolated microorganisms were be able to degrade laminaran into glucose up to 1516.59 mg/L with 30.33% yield after 3 days.

The HPLC analysis showed that product formed from degradation of laminaran was not only glucose but other sugars larger than glucose were also produced. It is indicating that enzyme from isolated microorganisms degraded laminaran in an endo-manner splitting or endo-enzyme.

According to the overall results, we found that the isolated microorganisms can be applied for polysaccharide degradation especially laminaran from brown seaweeds which would be important from the viewpoint of production of renewable fuels and promising for either seaweed industry or bioethanol industry.

However, the further study are still required to obtain an optimum results on biodegradation of laminaran.

V. Further Study

In the further study, more investigation about the effect of pH changes and interaction among six strains to obtain an optimum results on biodegradation of laminaran are still required. The production of organic acid (short chain fatty acid) and composition of degradation product formed form laminaran also need to be more analyzed.

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