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Production and Regulation of Gamma-aminobutyric Acid (GABA) Enriched Sea Tangle Extract by Lactic Acid Bacterial Fermentation

by

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February 26, 2016

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다시마 추출액에서 감마아미노뷰티르산 전환을 위한 유산균 발효조건

Advisor: Prof. Young-Mog Kim

by

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List of Abbreviation

GAD	**Glutamic acid decarboxylase**
LAB	**Lactic acid bacteria**
STE	**Sea Tangle extract**
MRS broth	**Man Rogosa Sharp broth**
CFU	**Colony Forming Units**
FAA	**Free amino acid**
GABA	**Gamma amino butyric acid**
HPLC	**High-Performance Liquid Chromatography**
NCBI	**National center for Biotechnology Information**
KCTC	**Korean Collection for Type Cultures**
M-MLV	**Moloney murine leukemia virus**
mRNA	**Messenger ribonucleic acid**
RT-PCR	**Reverse transcription-polymerase chain reaction**
cDNA	**Complementary DNA**
RNA	**Ribonucleic acid**

다시마 추출액에서 감마아미노뷰티르산 대량생산을 위한 유산균 발효조건

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요 약

다시마는 (*Saccharina Japonica*) 전통적으로 한국과 일본에서 식용으로 이용되어 왔고 항산화, 항균, 항당뇨, 함염 등의 효과가 있다고 보고 되어 있다. 선행 연구를 통하여 *Lactobacillus brevis* BJ 20 을 사용하여 다시마 발효 추출물에서 gamma amino butyric acid (GABA) 전환에 성공하였고, 이러한 GABA 가 풍부한 다시마 발효 추출액은 간기능 보호, 항산화, 항염증 등 다양한 생리활성을 가진다고 보고 되어 있다. 하지만 GABA 전환에 대한 gene regulation 에 대해서는 잘 알려져 있지 않아 본 연구에서는 GABA 전환을 극대화 하기 위해서 *L. brevis* BJ20 에 의해 glutamic acid 가 GABA 로 전환되는 gene regulation 에 대해 규명하기 위해 실험하였다. GABA 전환의 극대화를 위해, 유산균의 증식에 영향을 미치는 요소인 탄소원, 질소원, 유리 아미노산을 다시마 추출물에 첨가하여 균 증식 및 GABA 를 측정하였고, GAB 의 경우 HPLC 로 측정하였다. 그 결과, 탄소원의 경우 다시마 추출물에 glucose 1%을 첨가 하였을 경우 균의 증식은 유산균 증식 배지인 MRS broth 와 유사하였지만 GABA 는 생성되지 않았다. 질소원의 경우, 다시마 추출물에 3% yeast extract 첨가 하였을 경우 균의 증식이 MRS broth 와

유사하게 나타났고 GABA 전환 역시 6.36%로 가장 높게 측정 되었다. 이는 질소원에 있는 아미노산의 영향으로 판단 되어 GABA 전환에 대한 유리 아미노산의 영향을 분석하였다. 하지만, 다시마 추출물에 부족한 유리 아미노산을 첨가한 경우에도, GABA 의 전환은 나타나지 않았다. 또한, MRS broth 의 경우에도 거의 GABA 전환이 이루어지지 않았는데, 이는 MRS broth 에 있는 glutamic acid 의 함량이 낮은 것이 원인으로 분석 되었다. 이상의 결과를 종합해 보면, 다시마 추출물에 첨가한 yeast extract 가 glutamic acid 의 GABA 전환에 중요한 인자로 판단된다. 이에 GABA 전환에 관여하는 유전자 발현에 대한 yeast extract 첨가의 영향을 조사하였다

Yeast extract 를 함유한 다시마 추출액에서 발효 108 시간에 glutamate acid 가 GABA 로 거의 다 전환된 것으로 분석 되어, 이 조건에서 GABA 전환에 관여하는 유전자 발현을 RT-PCR 로 모니터링 하였다. 그 결과 GABA 전환에 관여하는 관련 유전자인 glutamate transcriptional regulator gene (*gadR*), gamma aminobutyric antiporter gene (*gadC*)와 glutamate decarboxylase gene (*gadB*)의 발현은 yeast extract 가 첨가된 다시마 추출물에서만 나타났다. 이상의 결과는 향후, 다시마 등의 해조류 발효를 통해 GABA 와 같은 bioactive compound 를 다량 함유하는 고부가가치제품의 개발을 위한 최적 발효조건 연구에 연결될 것으로 기대된다.

Introduction

Gamma aminobutyric acid (GABA) is an important compound of the free amino acid pool in both prokaryotic and eukaryotic organisms and it is also a representative depression neurotransmitter in the sympathetic nervous system (Roberts et al., 1950; Bazemore et al., 1957). GABA, a four-carbon non-protein amino acid, possesses well-known physiological functions such as neurotransmission, induction of hypotension effect, diuretic effect and tranquilizing effects (Jakobs et al., 1993; Somkuti et al., 2012). In addition, GABA showed several effects on treatments for sleeplessness, depression, and autonomic disorders (Okada et al., 2000), on treatment for chronic alcohol-related symptoms (Oh et al., 2003), and on stimulation of immune cells (Oh and Oh, 2003). It has been also reported that GABA is an insulin secretagogue from the pancreas and may prevent diabetic conditions (Adeghate and Ponery, 2002, Lee et al., 2010). There are studies on developing a functional food using GABA as an ingredient or additive in dairy products (Nomura et al., 1998; Park et al., 2006; Park et al., 2007; Siragusa et al., 2007), *Kimchi* (Lu et al., 2008; Pimentel et al., 1996; Seok et al., 2008), sourdough (Rizzello et al., 2008) and *Pocai* (Li et al., 2008).

GABA is primarily synthesized via decarboxylation of glutamate by glutamate decarboxylase (GAD), which has been found in bacteria such as

Pseudomonas aeruginosa (Noe and Nickerson, 1958), *P. fluorescens* (Tunnicliff. G, 1993), Marine *Pseudomonas* sp. isolates (Kaspar et al., 1991; Mountfort and Pybus, 1992), lactic acid bacteria (Siragusa et al., 2007), *Escherichia coli* (Richard and Foster, 2003), plants (Steward et al., 1949), and animals (Shelp et al., 1999; Snedden and Fromm, 1999; Kinnersley and Turano, 2000). GABA is commercially produced by fermentation method using bacteria (Yokoyama et al., 2002), fungi (Kono and Himeno, 2000) and yeast (Hao and Schmit, 1993). There are many reports on the ability of lactic acid bacteria (LAB) to produce GABA (Siragusa et al., 2007; Li and Cao, 2010). In addition, currently some studies have been progressed on the bio-synthetic production of natural GABA using natural resources such as raspberry juice (Kim et al., 2009), grape must (Di cargno et al., 2010), red seaweed (Ratanaburee et al., 2011), brown seaweed (Lee et al., 2010), soya yogurt (Park et al., 2007), and fishery products (Thwe et al., 2011).

Sea tangle (*Saccharina japonica*) is representative marine brown seaweed that is commonly used as seasonings, condiments and health food in Korea, Japan and China. There are many reports on various *in vitro* and *in vivo* biological activities of sea tangle on the antioxidant (Huang et al., 2004; Yuan et al., 2006; Park et al., 2009) and chemo preventive activity (Zhang et al., 2008). It has been previously reported that a production of GABA-enriched sea tangle extract (STE) by *Lactobacillus brevis* BJ-20 since the

edible brown seaweed contains abundant glutamate (Eom et al., 2010). The GABA-enriched STE exhibited enhanced activities on its antioxidant, anti-inflammatory and preventive hepatoprotective (Kang et al., 2013; Lee et al. 2010; Choi et al., 2012). Most of studies on GABA production were on the bioconversion of GABA using monosodium glutamate as a substrate (Lu et al., 2008). Recently, it was reported on the GABA conversion using STE (Lee et al., 2010; Kang et al., 2011). However, little information is available on factors effecting bioconversion of GABA by the lactic acid bacterial (LAB) fermentation in STE.

The aim of this study is to investigate optimization of GABA production and to elucidate the mechanism of GABA conversion by LAB fermentation. In the present study, it was reported the effect of various nutrients such as carbon sources, nitrogen sources and free amino acids on GABA production by LAB fermentation in STE. In addition, it was investigated the effect of the nutrients on the mRNA expression of genes related with GABA bioconversion; glutamate transcriptional regulator gene (*gadR),* gamma aminobutyric antiporter gene *(gadC),* and glutamate decarboxylase gene (*gadB*).

Materials and Methods

1. Preparation of sea tangle extract (STE)

Sea tangle (*Saccharina japonica*) was purchased at Gi-jang market, Busan, Korea in March 2015. Sea tangle was washed with fresh water to eliminate foreign materials such as sand, shells, and others. Then, the sample was added to water at a ratio of 1:5 (w/v) and agitating for 16 h. And then, STE was filtered with a filter (20 mesh) and finally, it was obtained 25 brix of STE. The STE stock was kept at -18°C before use.

2. Bacterial strains and medium

L. brevis BJ20 (KCTC 11377BP) was used as a starter. This strain was previously isolated from Korean traditional fermented food *Jot-gal* (cod gut) (Lee et al., 2010). The lactic acid bacteria was grown aerobically at 37°C in Lactobacilli MRS broth (Difco, Detroit, MI. USA) and subsequently used in experiments throughout this study. In order to ferment STE using *L. brevis* BJ20 strain, the stock culture was pre-cultured in Lactobacilli MRS broth

(Difco) at 37°C for 24 h to obtain an initial viable cell of approximately 10^9 CFU/mL. Then, the culture (approximately 10^3 CFU/mL of *L. brevis* BJ 20) was inoculated in STE.

3. Analysis of viable cells and pH

The number of viable cells was determined by the standard plate count agar method. Serial decimal dilutions of each sample were plated in duplicate onto MRS agar (Difco, Detroit, USA) plates and incubated at 37°C for 48 h before enumeration. The value of pH was determined three times by a pH meter (model AG 8603, Mettler-Teldo, Switzerland).

4. Lactic acid bacterial fermentation in STE

In order to investigate the effect of the nutrients on the growth of lactic acid bacteria and GABA production, *L. brevis* BJ 20 was inoculated in 3, 5, 7, and 9 brix STE containing various concentrations of carbon sources, nitrogen sources, free amino acids (FAA) and then stationary cultivated at 37°C. A common medium for lactic acid bacteria, Lactobacilli MRS broth, was used as a control. Samples were taken with regular intervals (every 12 h)

over the fermentation periods to determine number of viable cells, pH values and GABA contents.

5. Analysis of free amino acids (FAA) content and composition

In order to quantify FAA content, 1 mL of samples was filtered by 0.20 μm membrane filter (Toyo Roshi Kaisha Ltd, Tokyo, Japan). The analysis was done at the Center for Scientific Instrument, Gangneung-Wonju National University (Gangneung, Korea). The FAA analysis conditions are shown in Table 1.

6. Analysis of gamma aminobutyric acid (GABA)

GABA content was determined by a High Performance Liquid Chromatography (HPLC) equipped with C18 column (250 × 4.6 mm 5 μm, Dionex). Prior to analysis, each sample was diluted by 0.02 N hydrogen chloride (HCl) and filtered by 0.20 μm membrane filter (Toyo Roshi Kaisha Ltd., Japan). To determine GABA content, a standard solution containing

known concentration of GABA was analyzed along with samples in every series of analysis. The analysis conditions are shown in Table 2 and 3.

7. RNA isolation of *L .brevis* BJ20 cells and RT-PCR analysis

In order to investigate a regulatory mechanism in GABA bioconversion, the mRNA expression of GAD related genes (*gadR, gadC,* and *gadB*) was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR). The mRNA expression of *gadR, gadC,* and *gadB* genes was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR).

In order to extract total RNA, *L. brevis* BJ20 cells were grown at 37°C in STE containing various nutrients for 108 h. After fermentation, cells were harvested and then total RNA was isolated using zirconia beads and an RNAwiz kit (Ambion Inc.; Austin, TX, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the isolated RNA (1 μg) in a reaction catalyzed by SuperScript™II reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) with random hexanucleotides, according to the manufacturer's instructions. The synthesized cDNA was qualified and quantified by a VERSA Max

microplate reader, and the cDNA (200 ng) was used as a template for PCR.

Sequence-specific primers of each gene were designed based on the sequence of National Center for Biotechnology Information (NCBI) as shown in Table 4. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH gene) was used as a control. The RT-PCR condition was described in Table 5 and 6. Amplification was performed in a Thermal cycler (Takara Bio Inc., Shiga, Japan) with 30 cycles of denaturation at 94°C for 30 s, annealing at the indicated temperature of each primer for 30 s, and extension at 72°C for 30 s, respectively (Table 7 and 8). The amplified PCR products were analyzed electrophoretically on 1.5 % agarose gels and visualized with ethidium bromide.

8. Statistical analysis

All experiments were repeated three times. Data are expressed as the mean ±standard deviation for each measurement (n=3). The data of GABA and glutamate content obtained under different fermentation conditions were also analyzed by the analysis of variance (ANOVA) followed by the Duncan's multiple range test to determine whether there was any statistically significant difference ($P < 0.05$).

Table 1. Analysis conditions of free amino acids

Instrument	Hitachi L-8900 amino acid analyzer
Colum	Ion exchange column (4.6 mm X 60 mm)
Detector	UV length 570 nm
Wave length	Chanel 1 : 570 nm, chanel 2: 440 nm
Flow rate	Pump 1 : 0.35 mL/min, pump 2 : 0.3 m/min
Injection volume	20 μL
Temperature	Column oven temp : 30-70°C, Reaction coil temp : 135°C
Mobile phase	Pump 1 : buffer PF-1, PF-2, PF-3 PF-4 PF-RG Pump 2 : reaction reagents : Ninhydrin

Table 2. Operation conditions of HPLC analysis

Instrument	DINOEX Ultimate 3000 HPLC
Colum	C18 column (250 × 4.6 mm 5 μm, Dionex)
Detector	UV
Wave length	338 nm
Flow rate	1mL/min
Injection volume	20 μL
Temperature	Column oven temp : 40°C,
Mobile phase	Gradient A to B : A solvent : 50 mM Sodium acetate, pH 6.5 B solvent : Acetonitrile : MeOH : D.W. 45 : 45: 10 (v/v/v)

Table 3. Mobile phase conditions of HPLC analysis

Time (min)	solvent	
	A (%)	B (%)
0	90	10
12	60	40
13	10	90
17	10	90
17	90	10
23	90	10

Table 4. Gene-specific primers used for the RT-PCR

Gene	Direction	Sequence
GADR	Forward	5'- CAT ATG GAA TCC AGA ACT AAG G - 3'
	Reverse	5' - CTC GAG CTA GAC AGATFC CAA TTG - 3'
GADC	Forward	5' - CTA GCA GTT GCT GCG ATC TG - 3'
	Reverse	5' - GCT GAT TTC ACC CAA GAC AC - 3'
GAD1	Forward	5' - CAT TGG GAC CTC AAC TGT AG - 3'
	Reverse	5' - GCA TCG TCG CGG TCT TAT TA - 3'
GAPDH	Forward	5' - CTG CTA CGG ATA CGG GTA TC - 3'
	Reverse	5' - TGC AAG AAC CAG CAG AAA CG - 3'

Table 5. Chemicals used for RT-PCR

PCR Chemical	Amount used	Stock
M-MLV reverse transcriptase	1 μL	200 U/μL
dNTP mixture	1 μL	each 10 mM
Random Primer (6 mer)	1 μL	80 nM
DTT	2 μL	100 mM
5X First strand buffer	4 μL	
RNase out	1 μL	40 U/μL

Table 6. RT-PCR conditions

Temperature	Time	Cycle
94ºC	1 min	1
94ºC	30 sec	
55ºC	30 sec	30
72ºC	30 sec	
72ºC	5 min	1

Table 7. Chemicals used for PCR

PCR Chemical	Amount used (μL)	Stock
Ex-Taq polymerase HS.	0.2	5 U/μL
dNTP mixture	2	each 2.5 mM
Reverse Primer	1	10 pmole/μL
Forward Primer	1	10 pmole/μL
10X Ex-Taq buffer	2	
D.W	11.8	

Table 8. PCR conditions

Temperature	Time
25ºC	10 min
37ºC	50 min
70ºC	15 min
4ºC	∞

Results and Discussion

1. Effect of carbon sources on the cell growth and GABA production by *L. brevis* BJ 20

It has been reported that nutrient composition and culture conditions affect GABA production during microbial fermentation (Wang et al., 2003; Pimentel et al., 1996; Blanc et al., 1994). To determine the effect of various carbon sources on the growth of *L. brevis* BJ 20 and GABA production, 1%, 3% and 5% of each carbon source were added into 3 brix, 5 brix, 7 brix and 9 brix STE, respectively.

In case of without addition of carbon sources into 3 brix, 5 brix, 7 brix and 9 brix STE, there is no significant difference in the growth rate of *L. brevis* BJ20 ranging in about 8-9 log CFU/mL (Fig. 1). The cell number of 9-10 log CFU/mL was shown in control medium, Lactobacilli MRS broth. The growth was promoted by the addition of carbohydrates. The highest growth rate of *L. brevis* BJ20 was observed in 5 brix STE with 1% glucose followed by 5 brix STE with 3% glucose, and 5 brix STE with 5% glucose while the addition of fructose, galactose and maltose exhibited similar growth

compared to the growth of 3 brix and 5 brix STE. The cell number of 8-9 log CFU/mL was observed in 5 brix STE with 1% glucose after 36 h of fermentation. Also, the addition of carbohydrates such as fructose, maltose and galactose into 5 brix STE resulted in a slight growth (from Fig. 6A to Fig. 9A). However, the lactic acid bacterial growth was retarded or inhibited by the addition of carbohydrates into 3 brix, 7 brix and 9 brix STE (from Fig. 2A to Fig. 5A and from Fig. 10A to Fig. 17A). Thus, the addition of carbohydrates, especially glucose, into 5 brix STE enhanced the *L. brevis* BJ20 growth. Liew et al. (2005) also reported showed a slight positive effect of glucose on the cell number of *Lactobacillus rhamnous*. Furthermore, the concentrations of substrates are important for achieving high GABA yield (Yang et al., 2008).

However, no GABA conversion was observed in Lactobacilli MRS broth, STE and STE supplemented with carbohydrates. To contrary with the current study, Choi et al. (2006) reported that the addition of 1% glucose resulted in the highest GABA productions by the fermentation of *Lactobacillus buchneri* in Lactobacilli MRS broth. Li and Cao (2010) also reported that 1.25% glucose was the best carbon source for high production of GABA among of carbon sources including L-arabinose, ribose, D-xylose, galactose, glucose, fructose, maltose, melibbiose, a-methyl D-glucoside, N-acetyl D-glucosamine and gluconate. However, in this study, GABA production was

not affected by addition of various carbon sources in STE even when glucose is a carbon source. Considering above results, it was concluded that carbon sources will not be an important factor in bioconversion of GABA by the fermentation of *L. brevis* BJ20 in STE.

During the fermentation, the changes of pH value were also monitored. As generally known, the values of pH were decreased as the growth of lactic acid bacteria progressed (from Fig.2B to Fig. 9B). The pH values were ranged from 4.0 to 5.3 in STE and STE supplemented with carbohydrates. However, no change of pH was observed in 7 brix and 9 brix STE supplemented with carbohydrates since *L. brevis* BJ20 was not grown in the both STE medium supplemented with carbohydrates (from Fig. 10B to Fig. 17B).

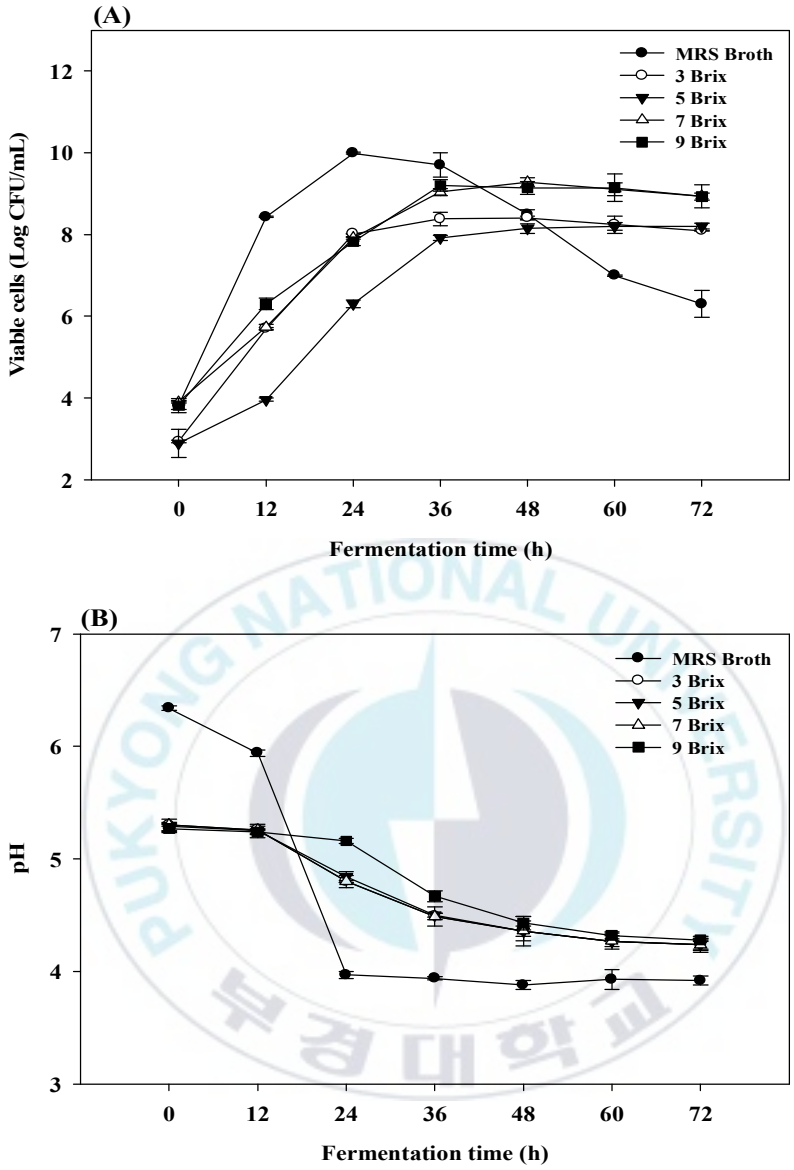

Fig. 1. Effect of Brix on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

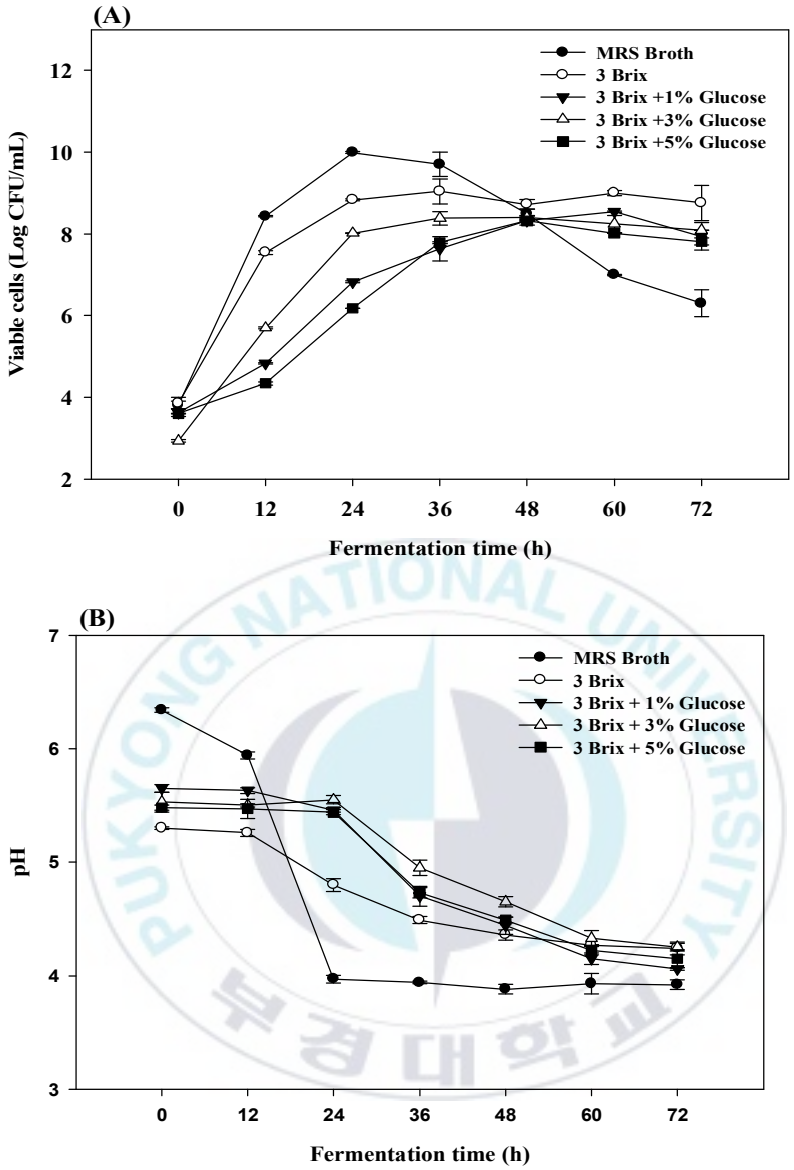

Fig. 2. Effect of glucose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

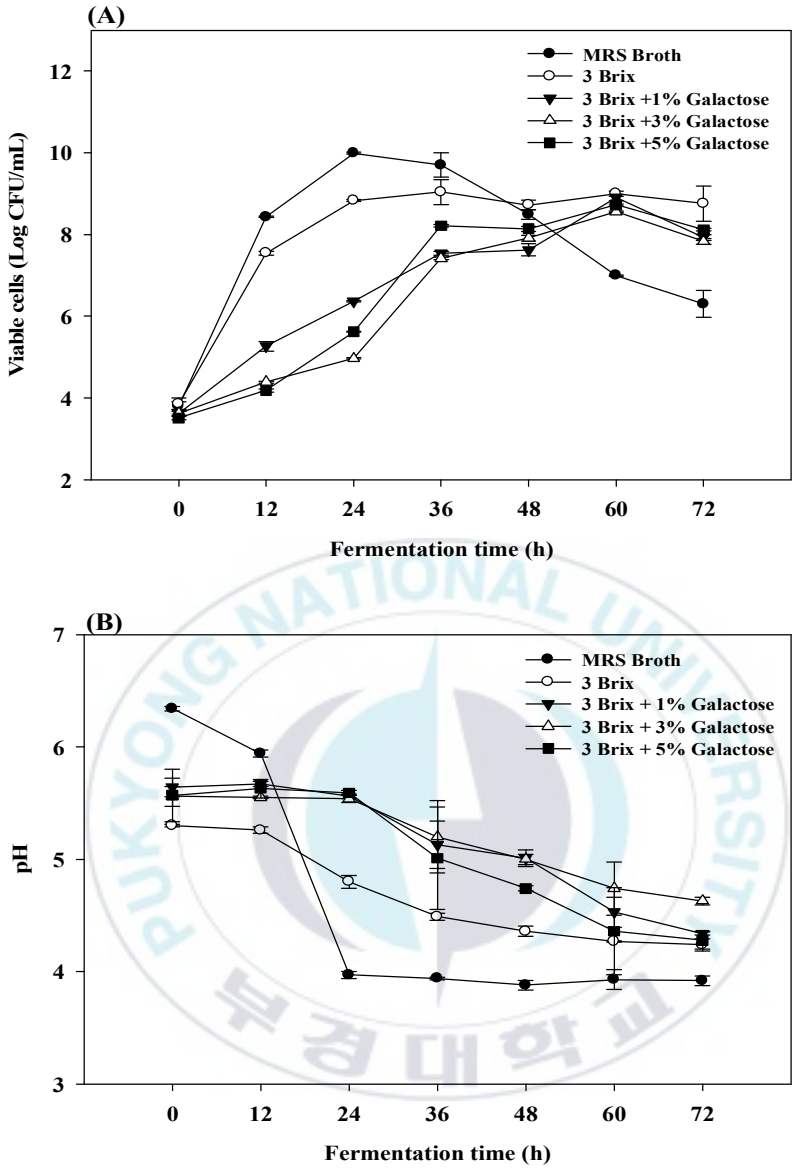

Fig. 3. Effect of galactose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

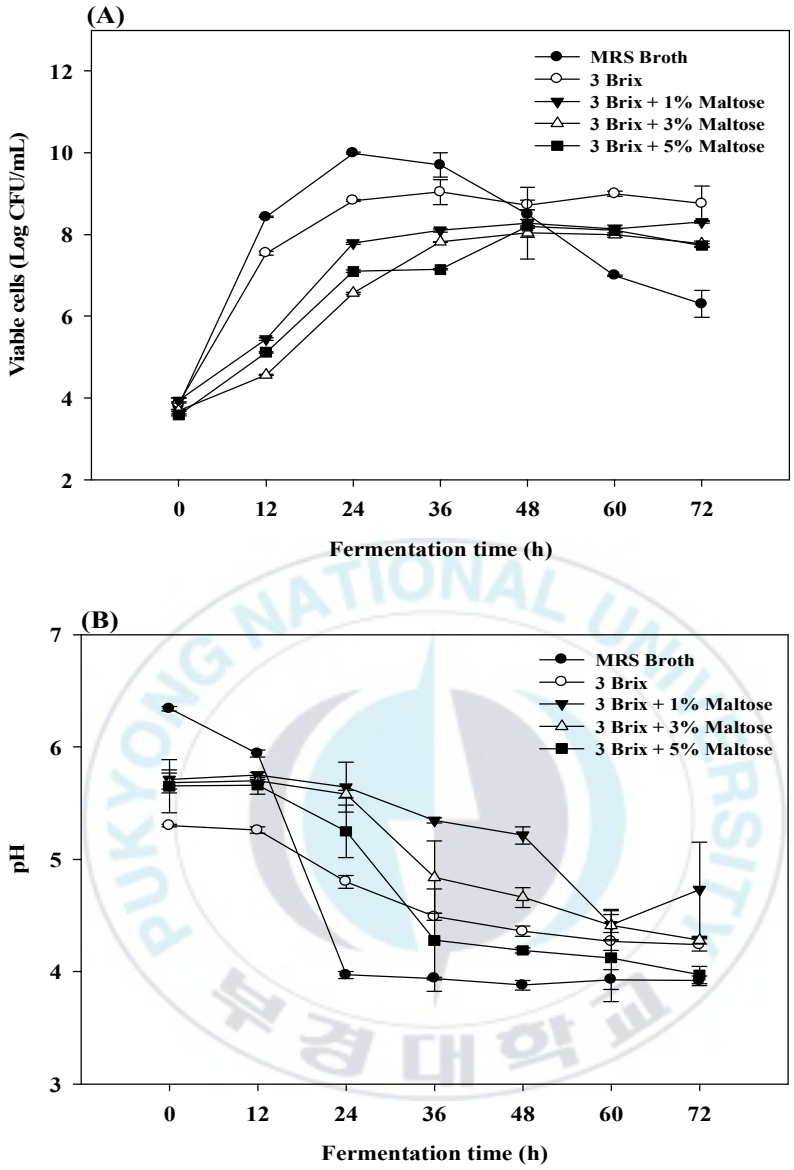

Fig. 4. Effect of maltose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

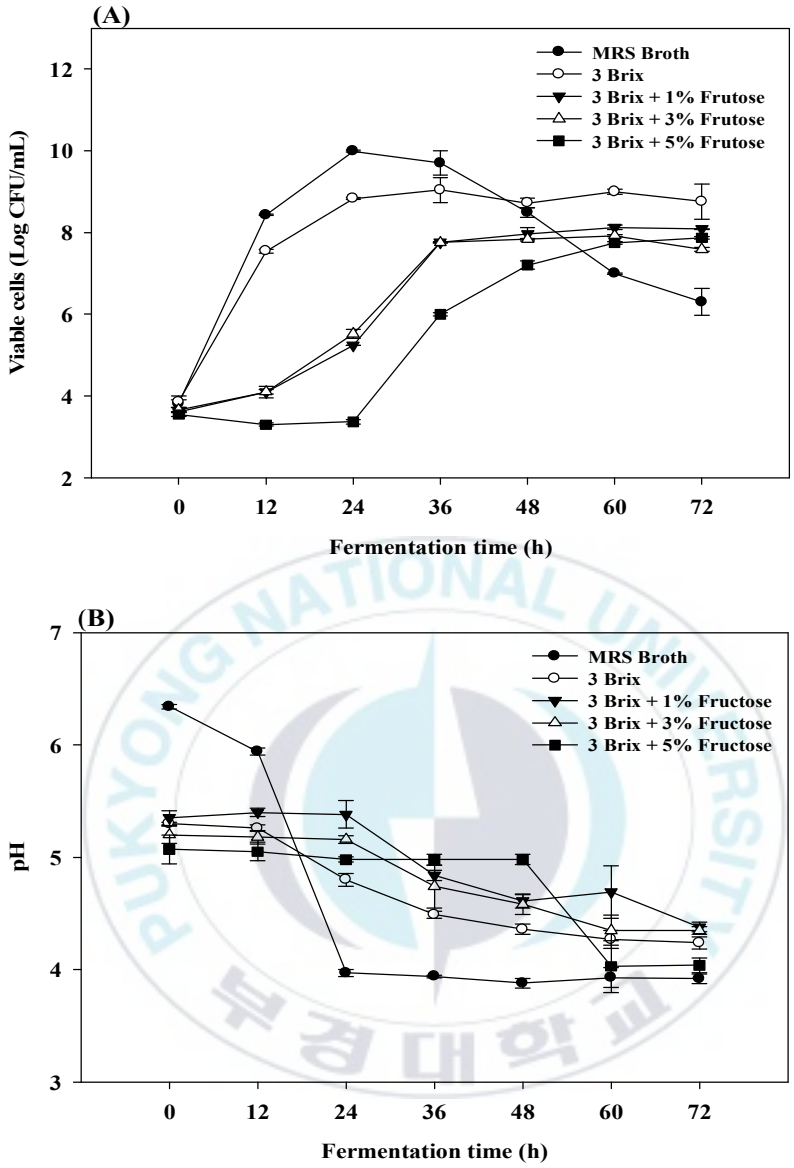

Fig. 5. Effect of fructose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

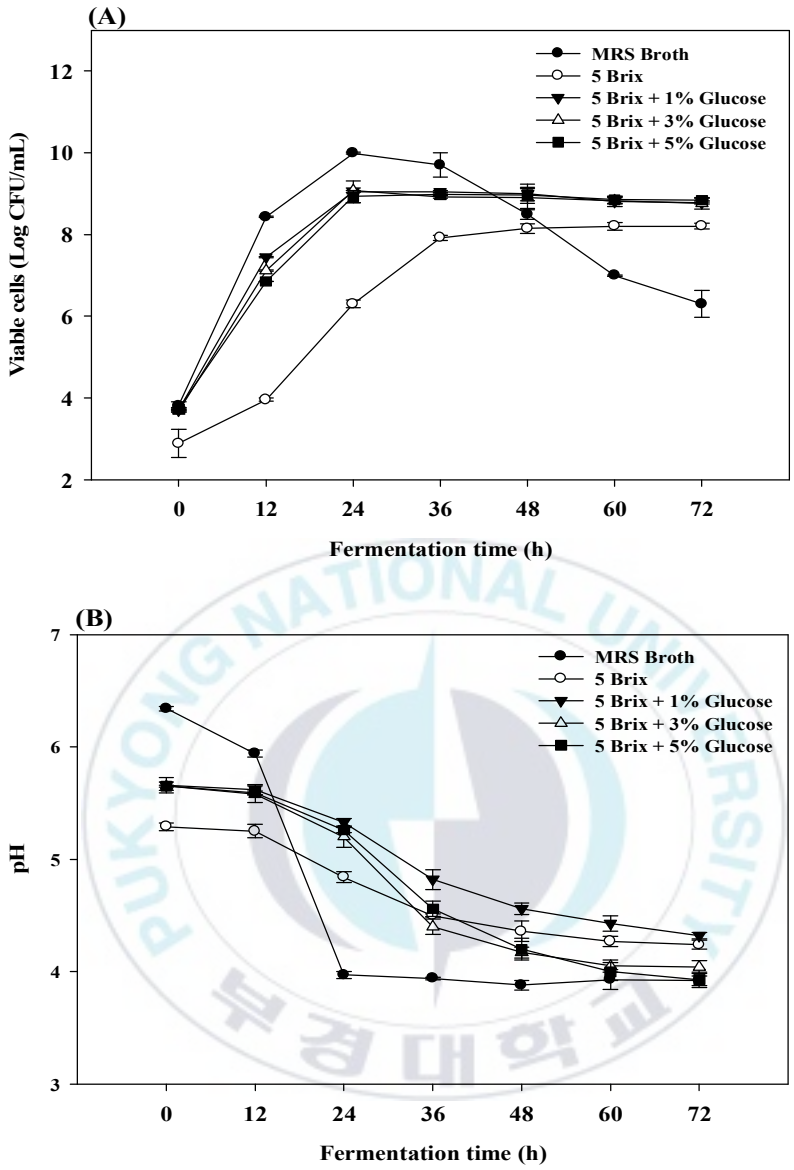

Fig. 6. Effect of glucose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

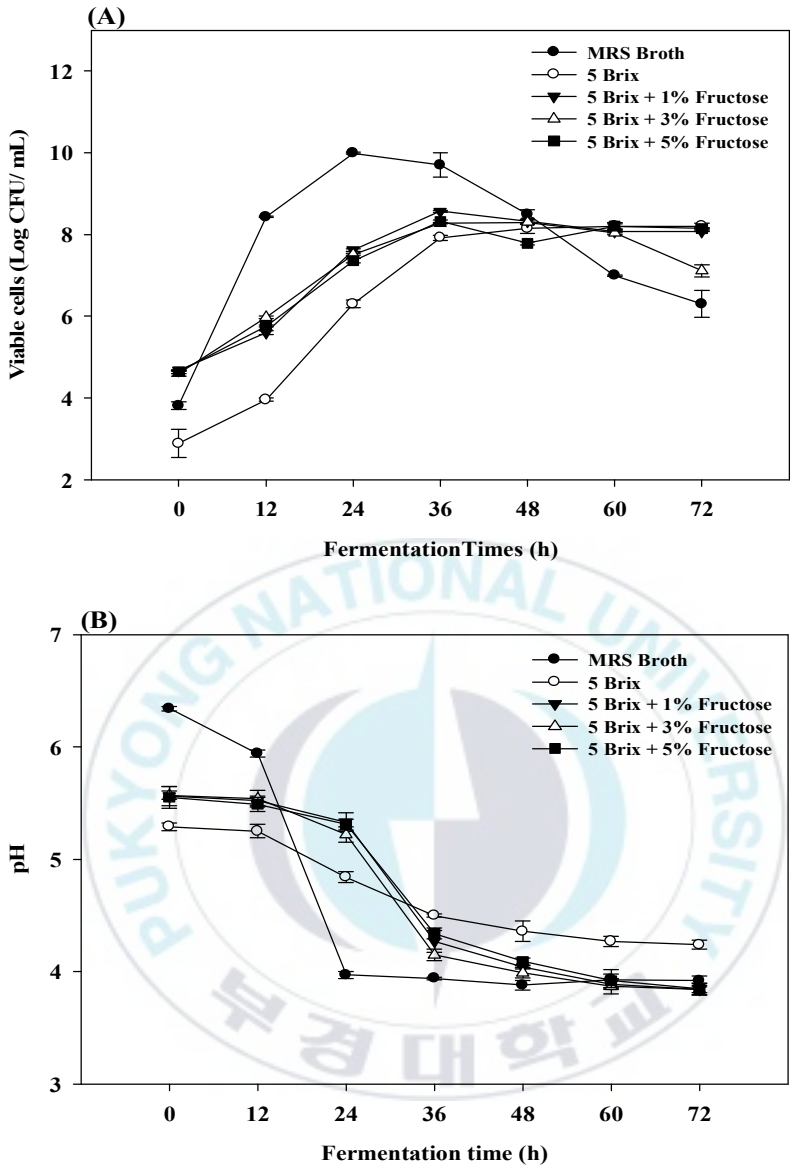

Fig. 7. Effect of fructose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

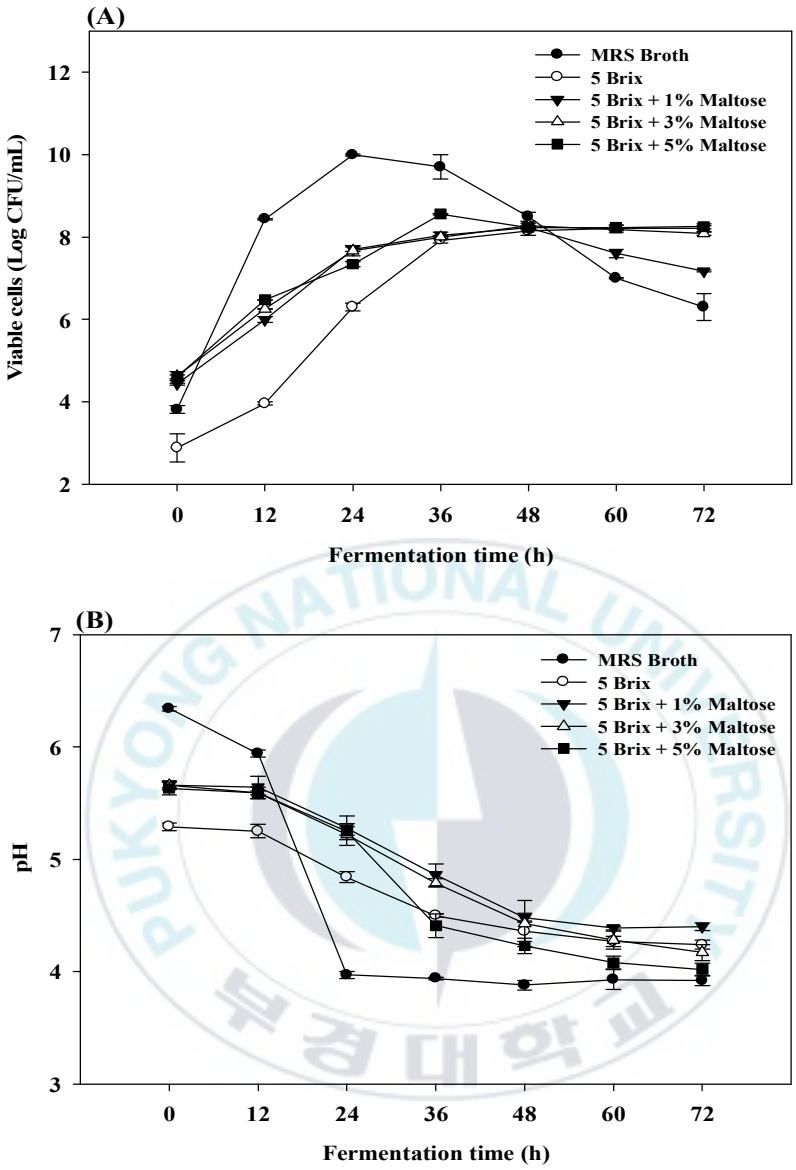

Figure. 8. Effect of maltose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

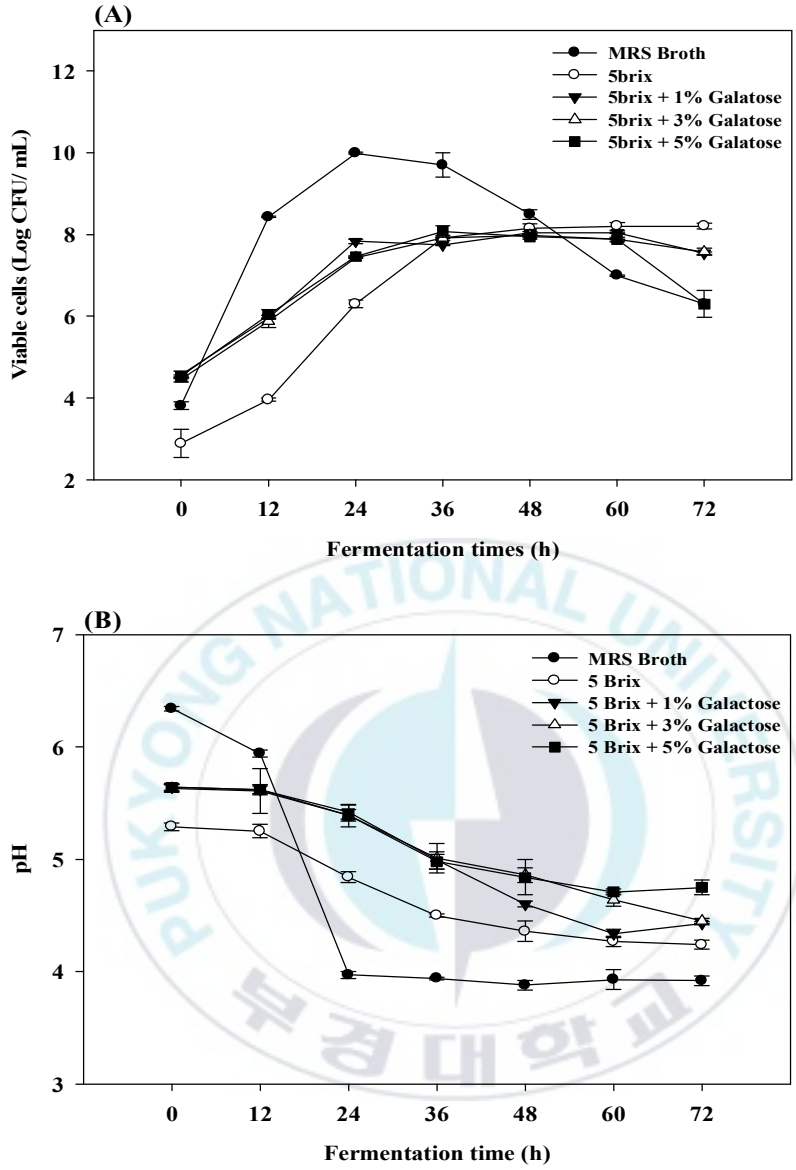

Fig. 9. Effect of galactose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

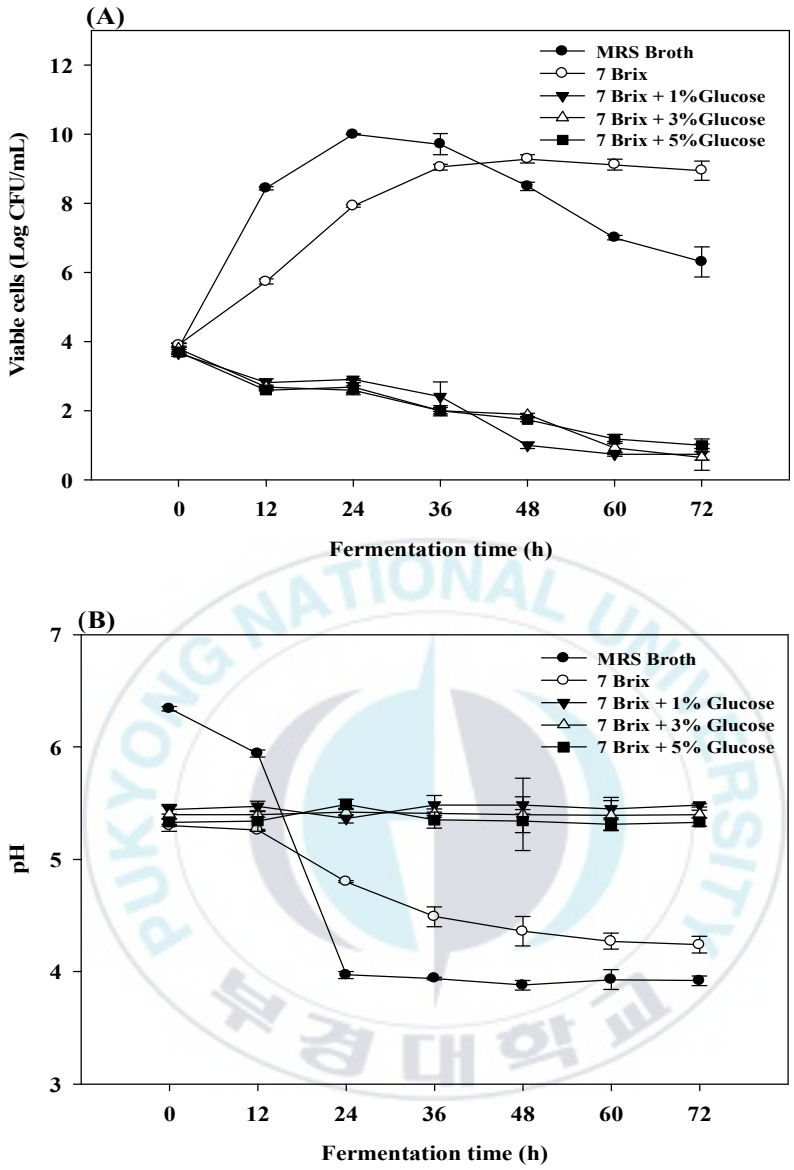

Fig. 10. Effect of glucose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 7 brix sea tangle extract.

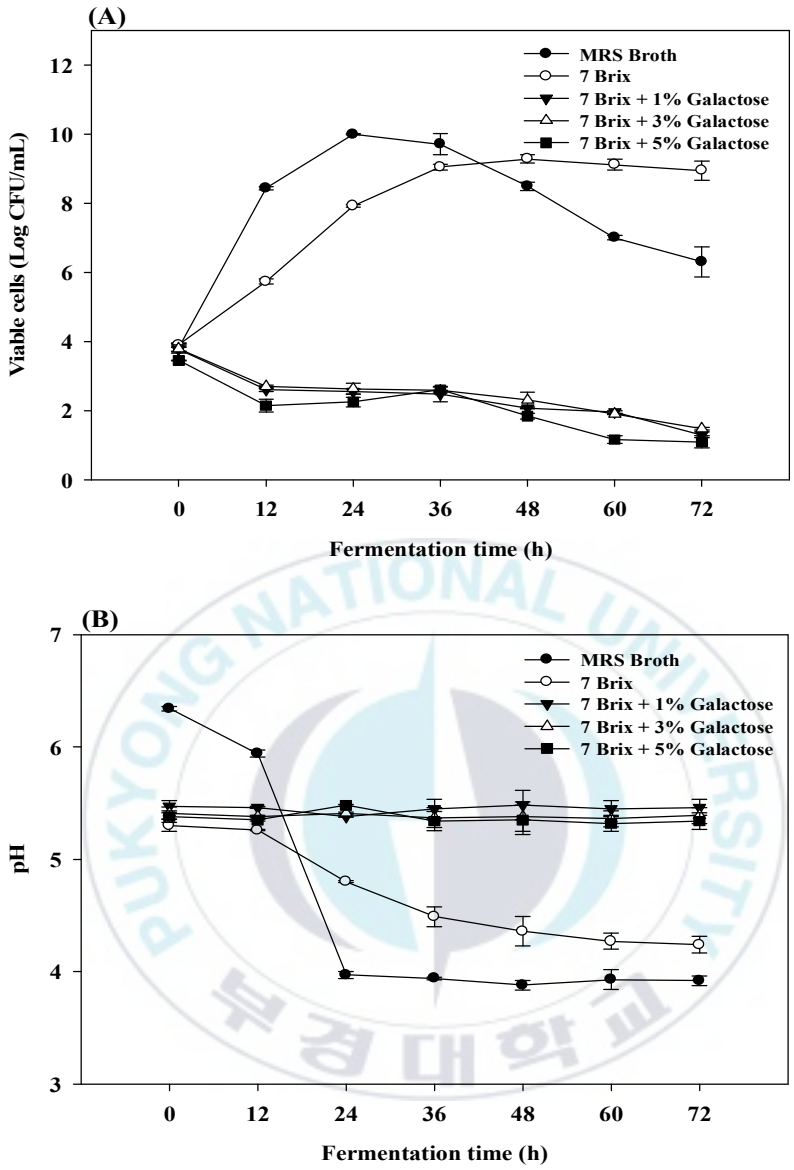

Fig. 11. Effect of galactose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 7 brix sea tangle extract.

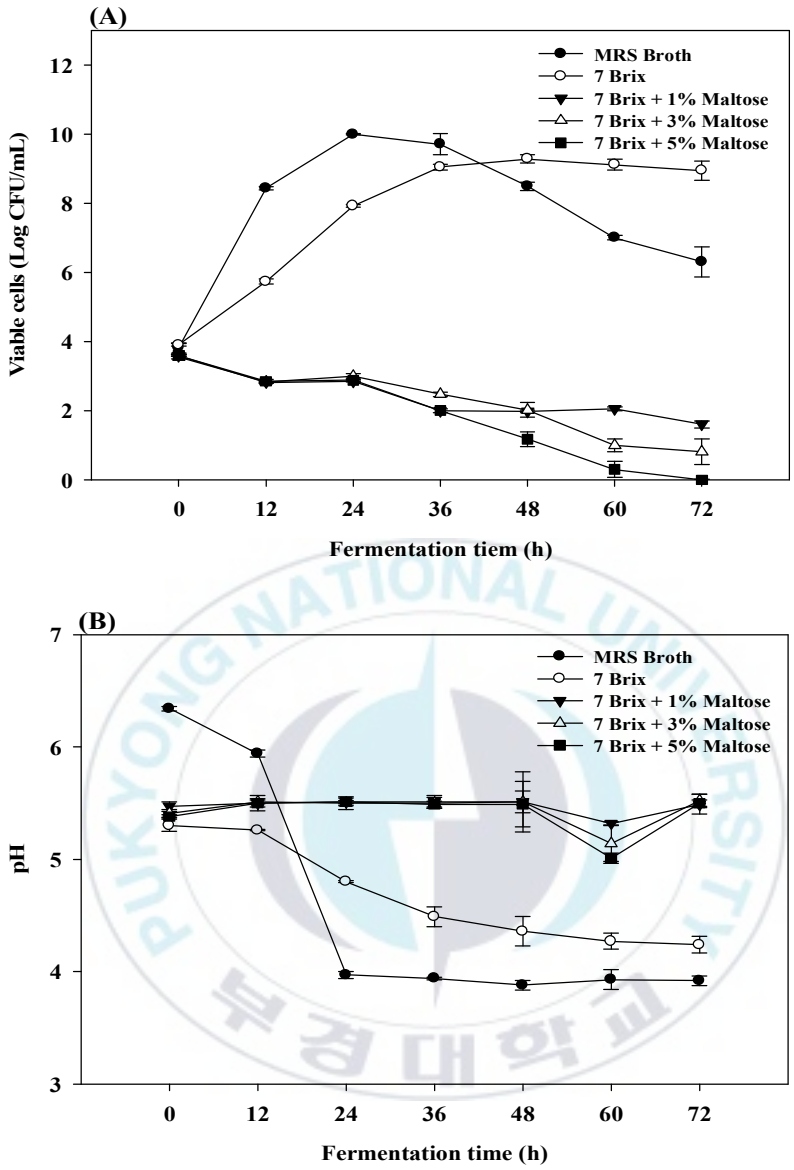

Fig. 12. Effect of maltose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 7 brix sea tangle extract.

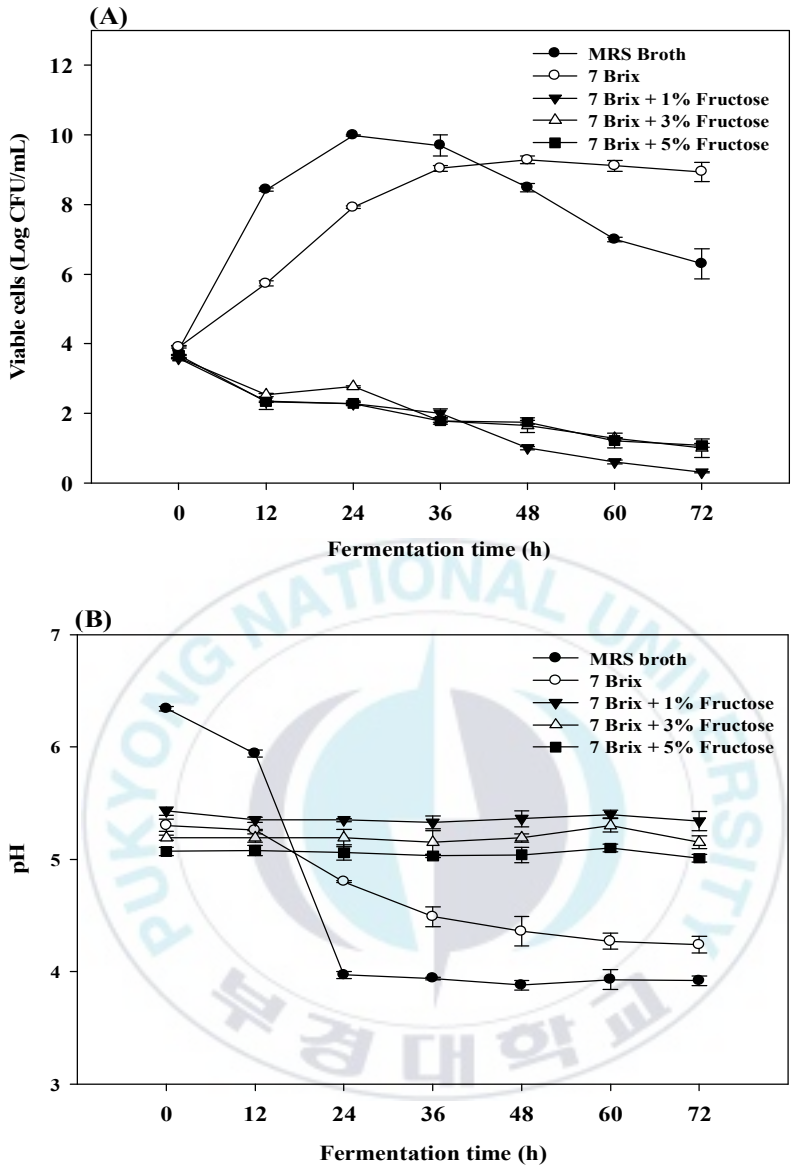

Fig. 13. Effect of fructose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 7 brix sea tangle extract.

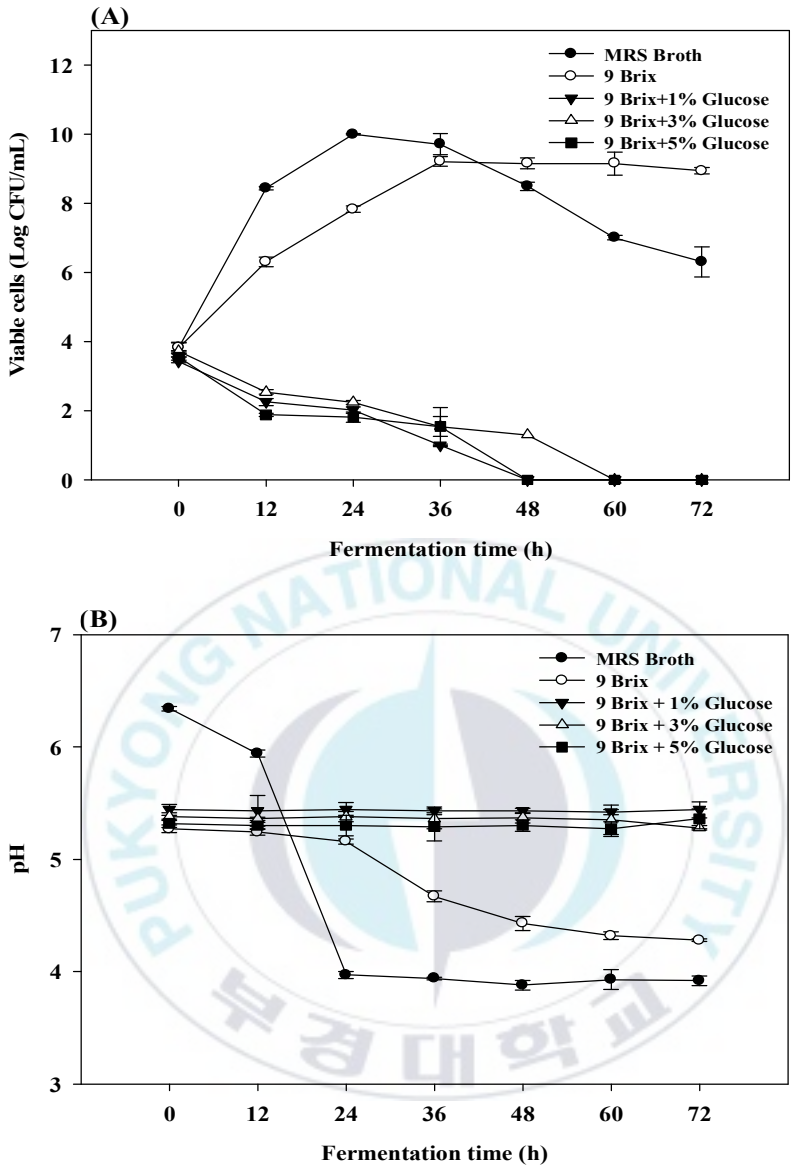

Fig. 14. Effect of glucose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 9 brix sea tangle extract.

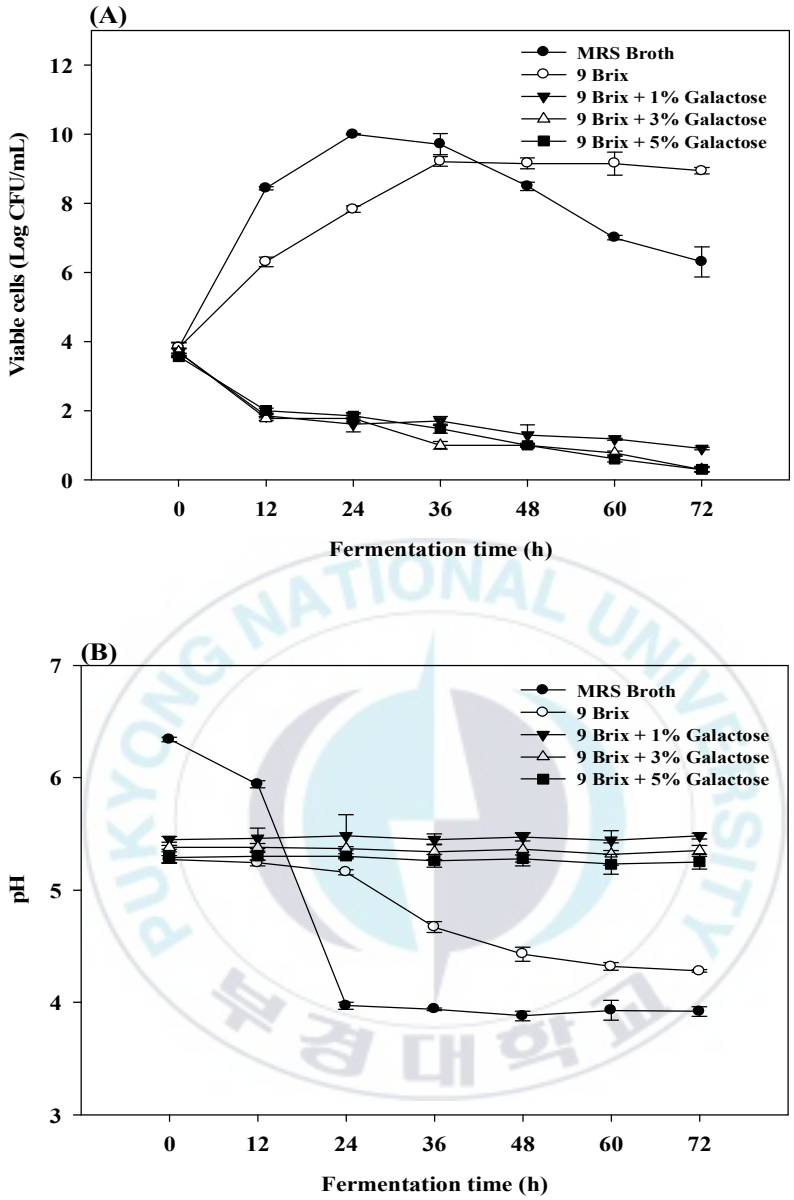

Fig. 15. Effect of galactose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 9 brix sea tangle extract.

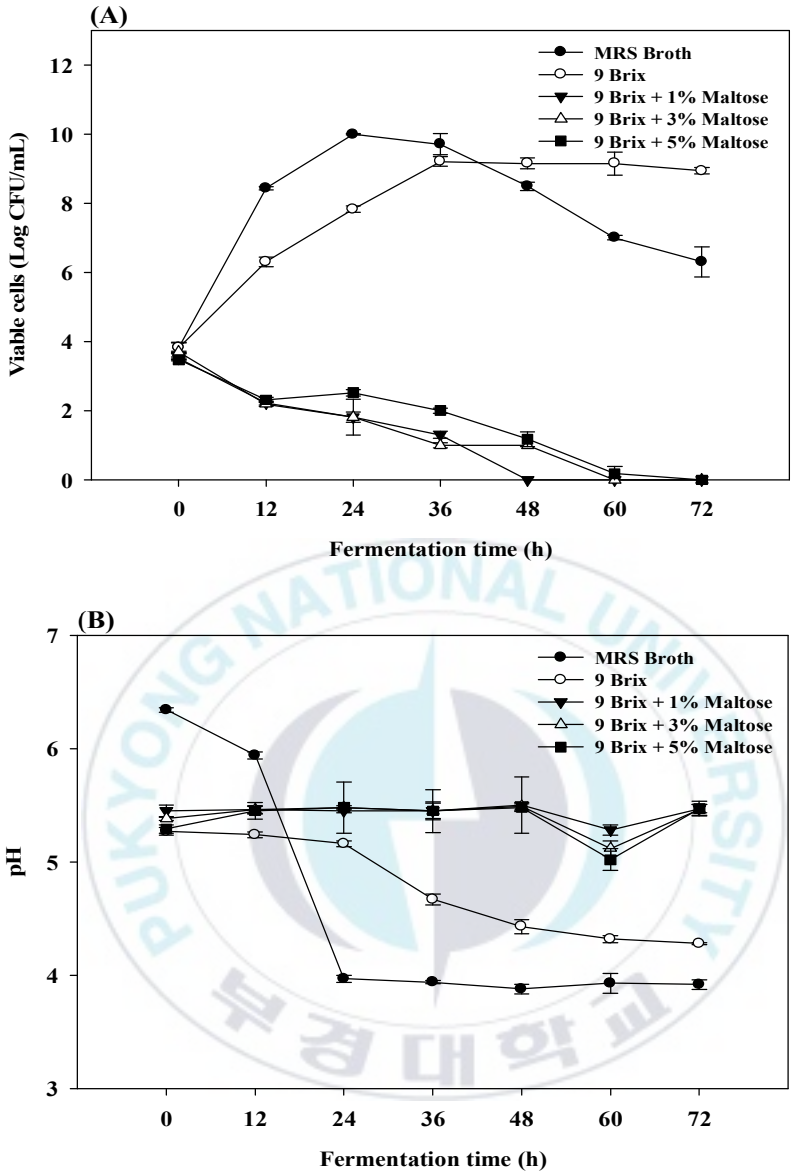

Fig. 16. Effect of maltose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 9 brix sea tangle extract.

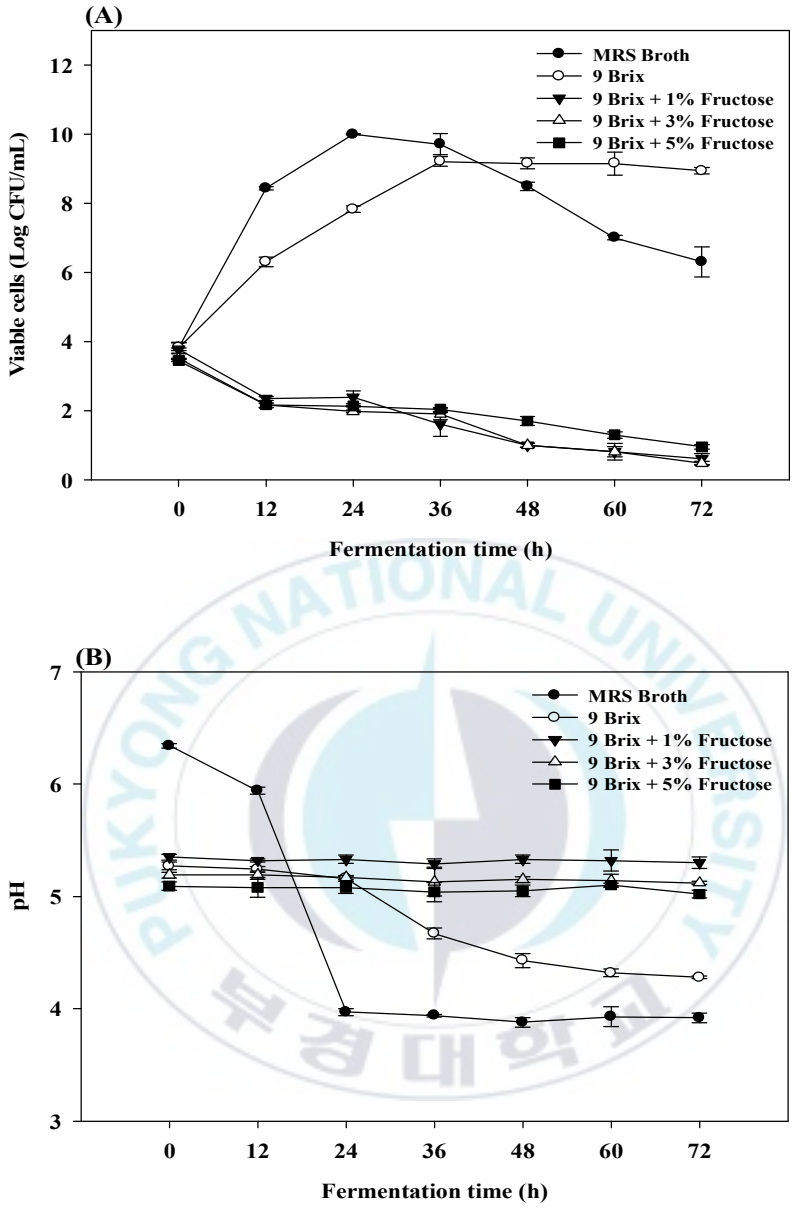

Fig. 17. Effect of fructose on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 9 brix sea tangle extract.

2. Effect of nitrogen sources on the cell growth and GABA production by *L. brevis* BJ 20

To determine the effect of various nitrogen sources on the growth of *L. brevis* BJ20 and GABA production, 1%, 3% and 5% of yeast extract were added into 3 brix, 5 brix, 7 brix and 9 brix STE, respectively. In case of addition of yeast extract in STE, there is no significant difference in the growth rate of *L. brevis* BJ20 ranging in about 8-9 log CFU/mL at 36 h of fermentation (from Fig. 18A to Fig. 21A). These results were almost similar with the growth rate observed in the control medium, Lactobacilli MRS broth. In general, the growth was increased by addition of yeast extract into 3 brix, 5 brix, 7 brix and 9 brix STE. Among them, 5 brix STE with 3% yeast extract was exhibited the highest growth rate of *L. brevis* BJ20, followed by 5 brix STE with 1% yeast extract, and 5 brix STE with 5% yeast extract (Fig. 19A). Based on these results, it was investigated the effect of other nitrogen sources such as meat extract and soy peptone on the cell growth of *L. brevis* BJ 20 in 5 brix STE medium. Also, the lactic acid bacterial growth was slightly enhanced, when the both nitrogen sources were added into 5 brix STE (Fig. 22A and Fig. 23A). However, these nitrogen sources exhibited less effect on the cell growth in STE than that of yeast extract. Thus, the

addition of 3% yeast extract into 5 brix STE medium resulted in the highest growth rate of *L. brevis* BJ20. Hujanen and Linko (1996) and Manteragas et al. (1995) also reported that the addition of yeast extract and peptone promotes the cell growth of lactic acid bacteria. Moreover, Fung et al. (2008) reported that meat extract, vegetable extract and peptone significantly influenced the growth of *Lactobacillus acidophilus*.

To investigate the effect of nitrogen sources on GABA production, 5 brix STE was used as a basal medium to evaluate an effect of GABA conversion, considering the highest growth rate. Samples were then obtained at 72 h of fermentation since the stationary growth phase of *L. brevis* BJ20 was observed at the fermentation point in all STE media supplemented with various nitrogen sources. In contrary to the addition of carbohydrates, GABA production was observed in all STE media supplemented with nitrogen sources, even though the GABA production was affected by the content of nitrogen sources (Fig. 24). Among of them, the highest GABA conversion was determined in 5 brix STE containing 3% yeast extract.

Next, it was investigate the effect of yeast extract and STE contents on GABA production. *L. brevis* BJ20 was inoculate into 3 brix, 5 brix, 7 brix and 9 brix STE supplemented with 1%, 3% and 5% yeast extract, respectably. Samples were also obtained after 72 h of fermentation. Among of them, the highest GABA production was determined in 5 brix STE

containing 3% yeast extract. Under the condition, the GABA content was about 6.3% flowed by 5 brix STE with 1% yeast extract (about 5.5% of GABA content) and 5 brix STE with 5% yeast extract (about 5.5% of GABA content) (Fig. 25). As describes above, however, GABA production was not determined under the condition of MRS broth only. As like the results obtained in this study, Li et al. (2008) and Sun et al. (2008) previously reported that the addition of nitrogen sources such as yeast extract, soya peptone and beef extract resulted in the GABA production in Lactobacilli MRS broth.

The effect of nitrogen sources on changes of pH value was also measured during the fermentation. As it is well known, the values of pH were decreased as the growth of lactic acid bacteria progressed. Interestingly, the pH values were slightly increased since 48 h later of fermentation in 3 brix, 5 brix and 7 brix STE supplemented with yeast extract (Fig. 18B, Fig.19B and Fig. 20B). Among them, the highest pH value, pH 5.81, was observed at 72 h of fermentation in 5 brix STE with 3% yeast extract (Fig. 19B). At this point, the highest GABA production was determined as described above. Thus, the increased pH value will be closely related with the GABA production in the medium. According to the report by Small and Waterman (1998), the decarboxylation reaction of glutamate mediated by GAD enzyme consumes a cytoplasmic proton, thereby generating a pH gradient across the

membrane. Consequently, GABA converted in a cell is transported into the outside of cell by an antiporter. As the resulting, the extracellular environment is to keep in a more alkaline. Thus, the pH was gradually increased as the GABA production progressed.

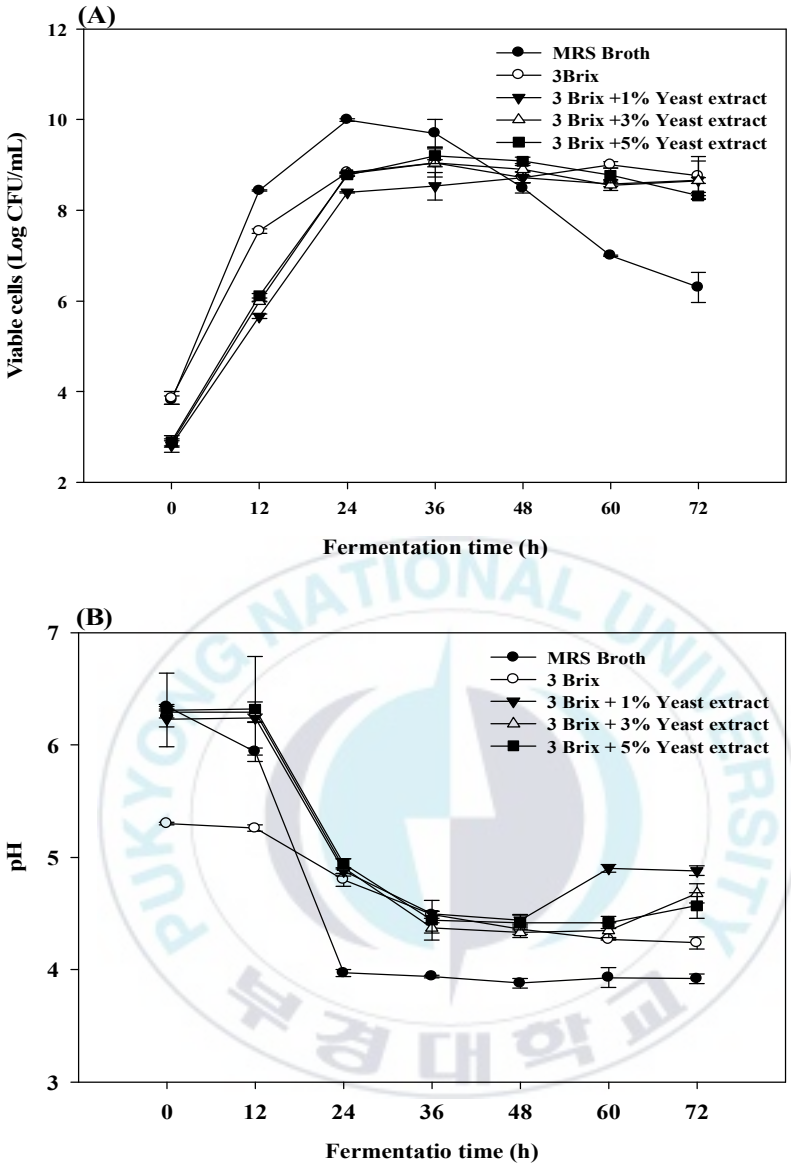

Fig. 18. Effect of yeast extract on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 3 brix sea tangle extract.

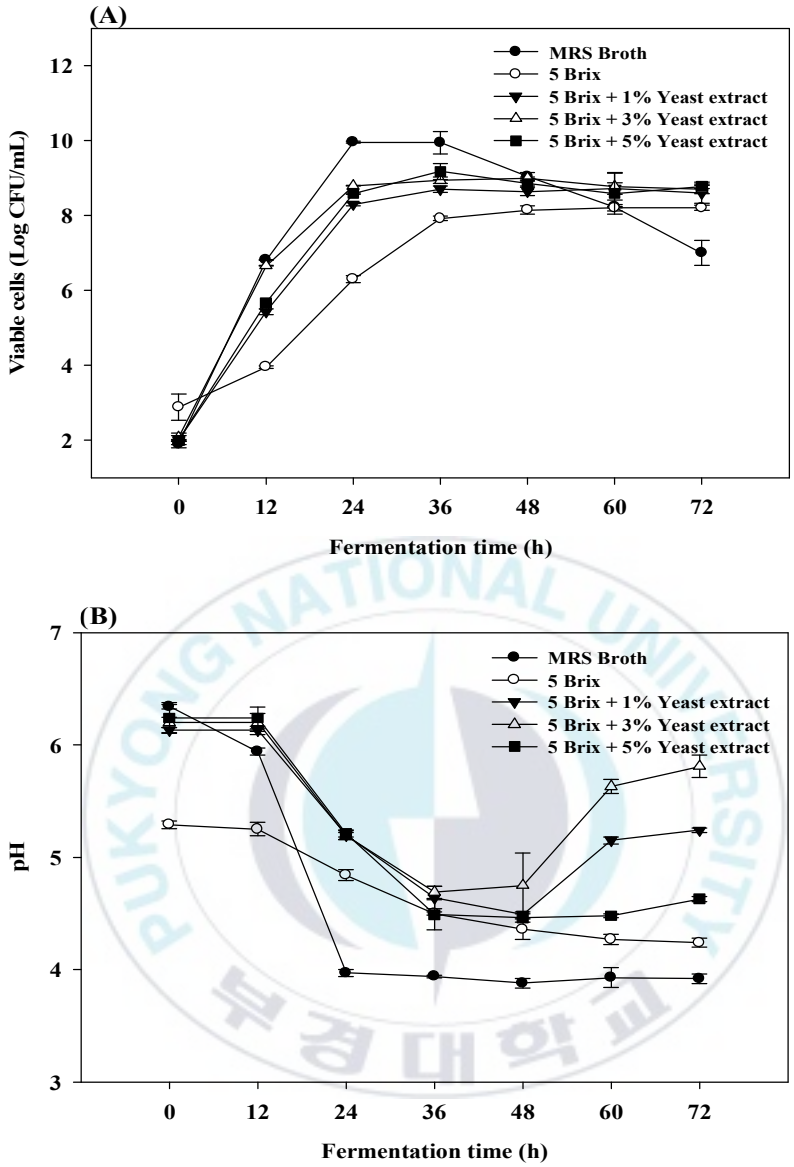

Fig. 19. Effect of yeast extract on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

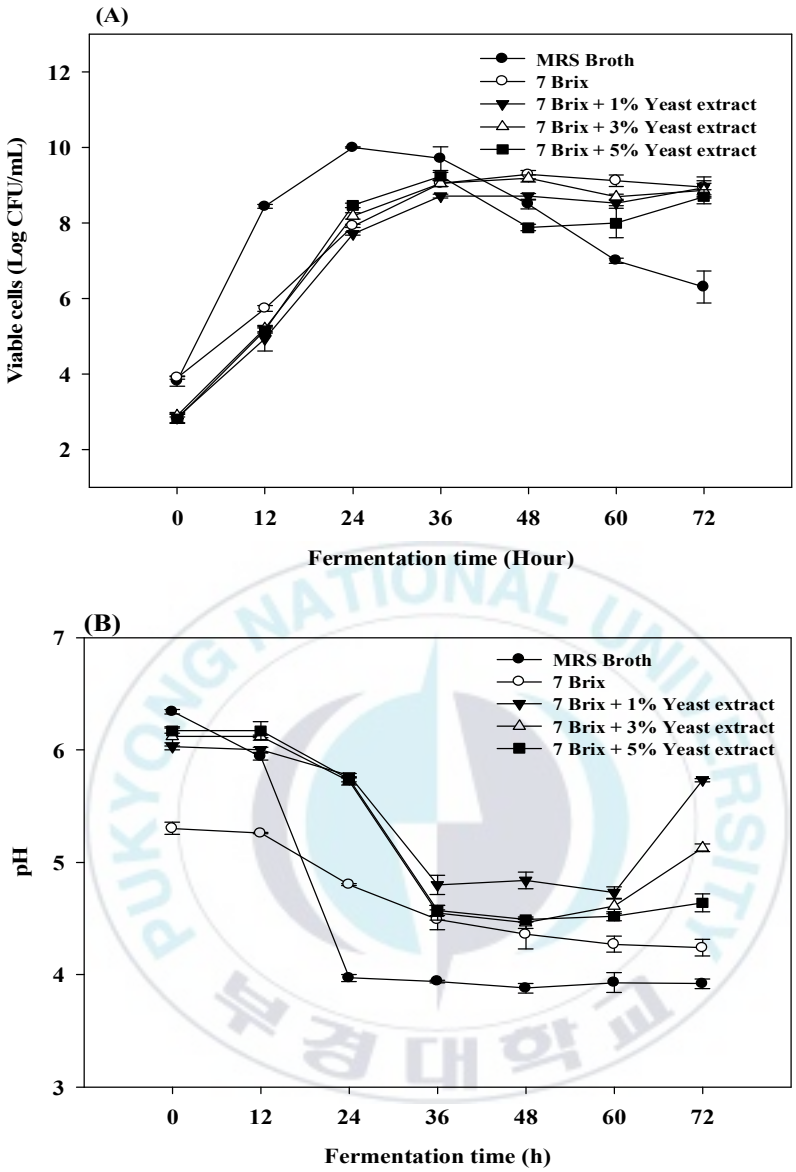

Fig. 19. Effect of yeast extract on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 7 brix sea tangle extract.

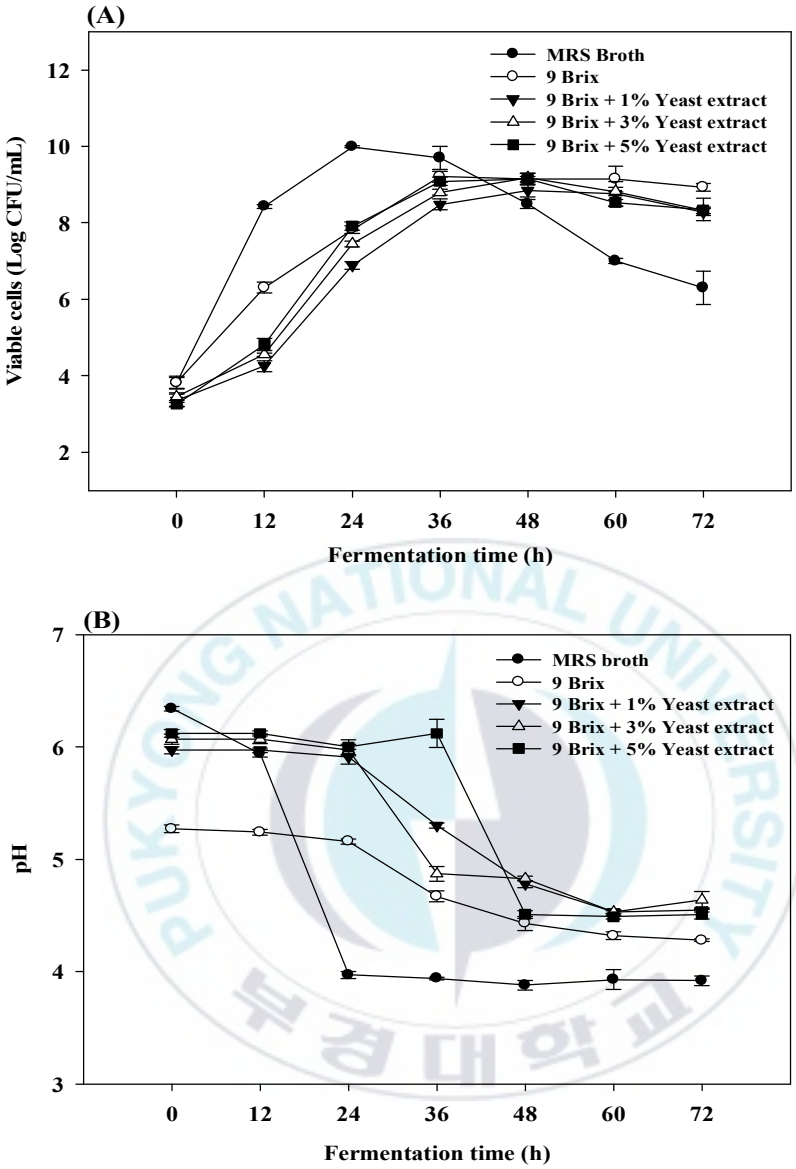

Fig. 21. Effect of yeast extract on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 9 brix sea tangle extract.

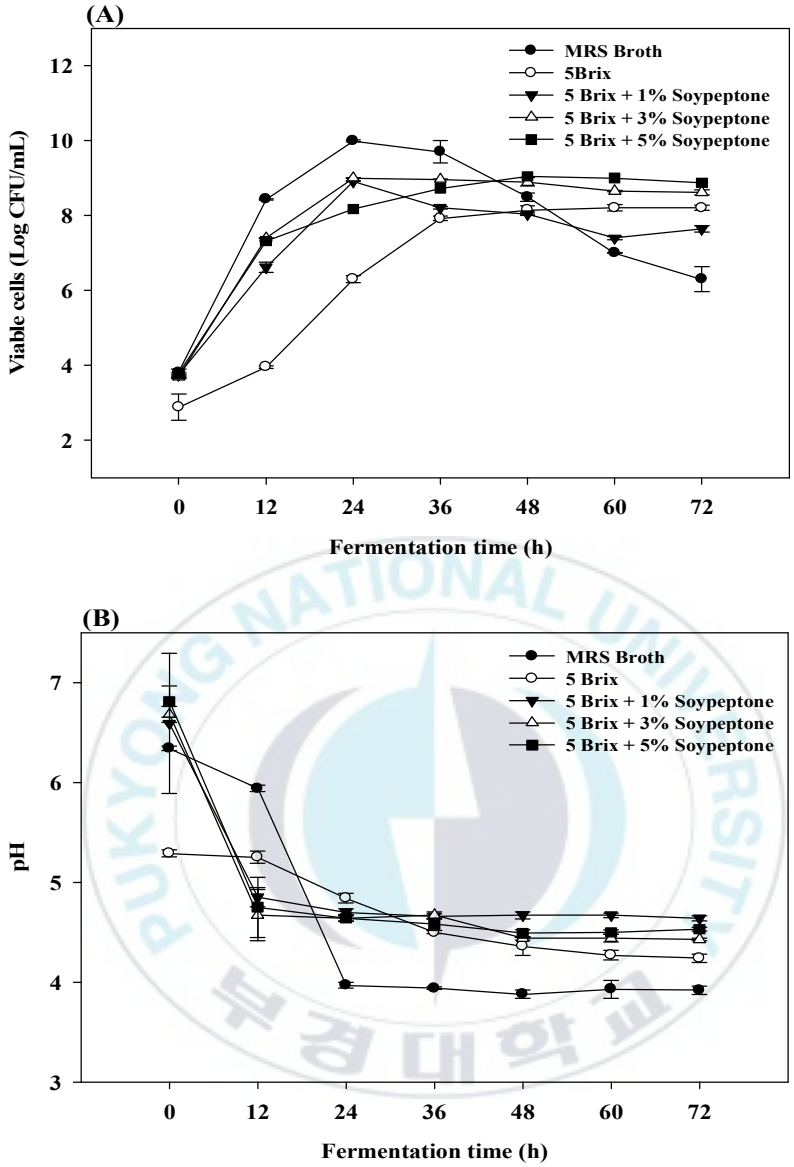

Fig. 22. Effect soy peptone on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

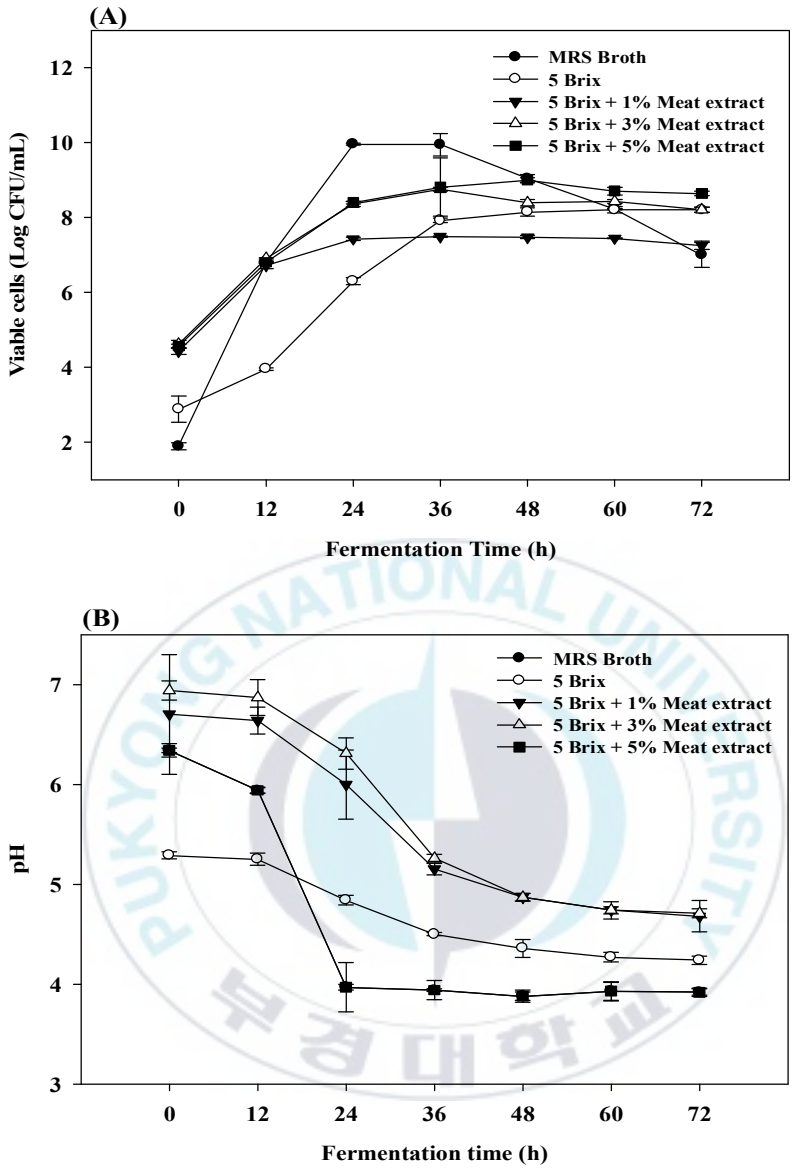

Fig. 23. Effect of meat extract on the growth of *Lactobacillus brevis* BJ20 (A) and on the change of pH value (B) in 5 brix sea tangle extract.

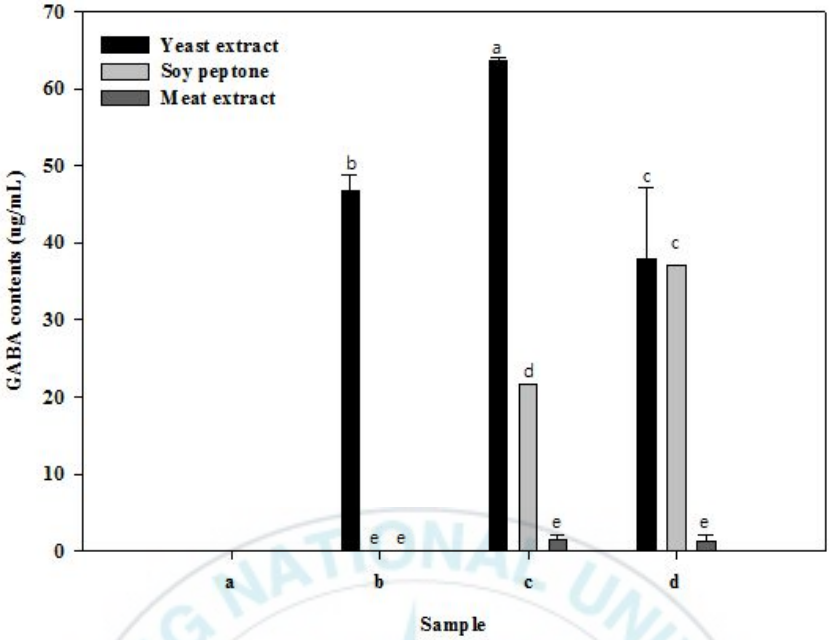

Fig. 24. Effect of nitrogen sources on GABA production by *Lactobacillus brevis* BJ20 fermentation in 5 brix sea tangle extract.

L. brevis BJ20 was fermented at 37°C for 72 h with stationary. Data represent the mean values for three replicate experiments. Bars represent standard deviations ($p<0.05$). (*: Not detected, a: 5 brix STE. b: 5 brix with 1 % nitrogen sources, c: 5 brix STE with 3 % nitrogen sources, d: 5 brix with 5 % nitrogen sources).

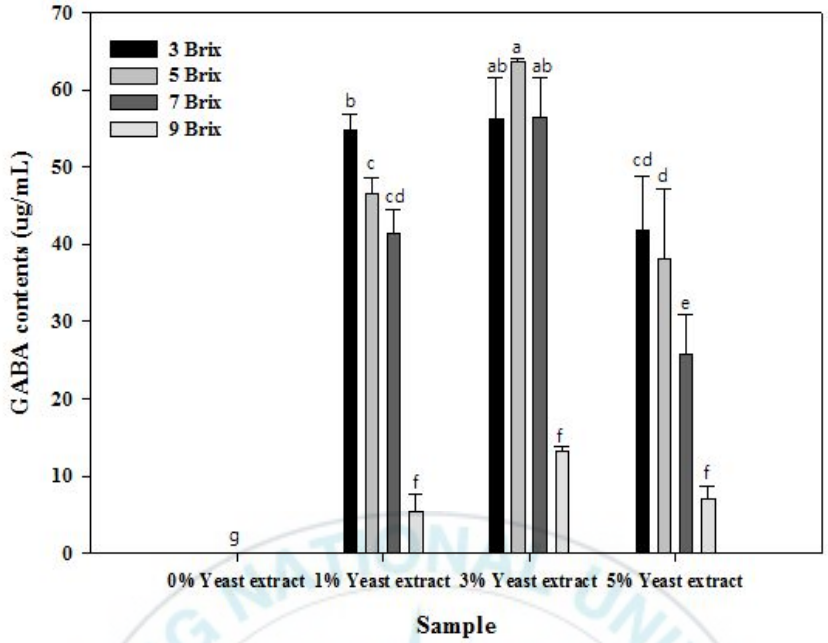

Fig. 25. Effect of yeast extract and sea tangle extract contents on GABA production by *Lactobacillus brevis* BJ20 fermentation.

L. brevis BJ20 was fermented at 37°C for 72 h with stationary. Data represent the mean values for three replicate experiments. Bars represent standard deviations ($p<0.05$).

3. Effect of free amino acids on the cell growth and GABA production by *L. brevis* BJ20

As shown in Fig. 24, the highest GABA production was observed in 5 brix STE with 3% yeast extract, while no GABA production was in 5 brix STE. These results indicated a possibility that amino acids will be a factor affecting GABA production in 5 brix STE. To investigate the effect of free amino acids on the growth of *L. brevis* BJ20, pH value and GABA productions, the contents of free amino acids were analyzed in Lactobacilli MRS broth, 5 brix STE and 5 brix STE with 3% yeast extract. In 5 brix STE supplemented with 3% yeast extract, there are significantly difference in the contents of free amino acids, compared to Lactobacilli MRS broth and 5 brix STE. The 5 brix STE with 3% yeast extract was shown higher amount of free amino acids than those of Lactobacilli MRS broth and 5 brix STE (Table 9). The analysis revealed that some amino acids including taurine, aspartic acid, threonine, glycine, methionine, leucine, tyrosine, phenylalanine, β-alanine, lysine, arginine, serine, valine, isoleucine and alanine, were insufficient amino acids amount in Lactobacilli MRS broth and 5 brix STE.

These insufficient amino acids were added into 5 brix STE and then the GABA production was monitored. In all media, the cell growth of *L. brevis* BJ20 was ranged in 7-8 log CFU/mL. There was no significant difference on the cell growth, suggesting that free acids have less effect on the bacterial growth in STE medium (Table 10). These results were also consistent with the report that the supplementation of amino acids exhibited no positive effect on bacterial growth (Polak-berecka M et al., 2010). In addition, no GABA production was determined in 5 brix STE supplemented with the insufficient free amino acids (Table 10). In contrast with the current study, however, Li et al. (2013) reported the free amino acids had positive effect on GABA biosynthesis in Brassica plants. It was thought that these phenomena were mainly from the difference on bacterial strain, culture medium and others.

In case of PH values, no significant changes of pH value were observed between the amino acids supplemented into STE. The pH values were ranged from 4.6 to 5.9 in 5 brix STE supplemented with free amino acids (Table 10).

Table 9. Analysis of free amino acid contents in media used in this study

(μg/mL)

Amino acid	Sample		
	Lactobacilli MRS broth	5 Brix	5 Brix + 3% yeast extract
Phosphoserine	62.10±24.10	12.21±0.21	68.26±1.2
Taurine	62.55±15.56	7.34±4.26	41.61±1.06
Phospho ethanol amaine	75.49±67.35	*	50.88±1.52
Urea	*	*	*
Aspartic acid	218.67±40.14	1604.91±57.54	2190.17±3.03
Threonine	146.73±27.42	7.06±2.99	452.44±0.86
Serine	183.91±30.94	7.11±10.97	590.60±0.73
Glutamic acid	525.14±75	3273.04±180.60	5788.09±5.62
Sarcosine	*	*	9.08±0.19
α-amino adipic acid	*	*	*
Glycine	112.53±16.04	10.71±2.13	358.15±0.6
Alanine	333.486±47.81	141.13±3.57	1288.57±1.844
α-amino-n-butyric acid	10.10±1.53	1.48±0.11	13.7±0.14
Valine	253.74±58.85	8.17±1.88	762.49±1.25
Methionine	118.84±17.10	*	210.28±0.07
Cystathionine	*	3.99±1.98	*
Isoleucine	223.29±57.27	2.37±1.66	587.72±0.83
Leucine	565.27±103.86	3.23±1.98	1158.30±1.85
Tyrosine	185.773±27.91	2.7±1.88	229.74±0.59

***:not determined.**

Table 9. continued

(μg/mL)

Amino acid	Sample		
	Lactobacilli MRS broth	5 Brix	5 Brix + 3% yeast extract
Phenylalanine	400.4±71.81	4.22±2.34	722.12±3.67
β-Alanine	65.34±12.83	6.22±0.67	33.57±3.11
β-Amino isobutyric acid	131.38±19.3	*	72.98±4.49
γ-Amino-n-butyric acid	53.23±12.39	1.72±0.18	40.18±8.64
Ethanol amine	15.03±10.18	12.27±1.35	44.75±11.83
Ammonia	142.21±23	28.75±13.31	76.06±7.78
Ornithine	15.75±3.13	2.75±0.48	79.25±0.14
Lysine	276.09±77.98	3.00±2.15	533.87±1.24
Histidine	34.5±5.07	*	118.61±0.11
Anserine	58.87±46.72	*	78.55±1.17
Carnosine	*	*	*
Arginine	212.29±37.45	2.01±2.43	453.14±1.27
Total	4551.03 ± 700.6	5174.9± 168.36	16053.5 ±54.24

***:not determined.**

Table 10. Effect of amino acids supplemented into 5 brix sea tangle extract on the growth of *Lactobacillus brevis* BJ20, pH values, and GABA production

Free amino acid	viable cell (log CFU/mL)	pH	GABA production
Taurine	7.76 ± 0.05	4.84±0.33	-*
Aspartic acid	7.89 ± 0.08	4.85±0.01	-
Threonine	7.38 ± 0.01	5.48±0.18	-
Glycine	7.59 ± 0.09	5.24±0.52	-
Methionine	7.81 ± 0.13	4.80±0.35	-
Leucine	7.73 ± 0.07	5.07±0.48	-
Tyrosine	7.40 ± 0.06	5.01±0.51	-
Phenylalanine	7.30 ± 0.11	5.54±0.09	-
β-Alanine	8.04 ± 0.03	4.64±0.08	-
Lysine	7.69 ± 0.06	5.67±0.97	-
Arginine	7.78 ± 0.03	5.85±0.81	-
Serine	8.33 ± 0.04	5.80±0.35	-
Valine	8.02 ± 0.01	5.90±0.51	-
Isoleucine	7.83 ± 0.05	5.60±0.03	-
Alanine	8.83 ± 0.72	5.60±0.02	-

The number of viable cell, pH value and GABA content were determined at 72 h of fermentation. *:not determined.

4. Effect of fermentation time on GABA production

As shown in Fig. 23 and Fig. 24, the highest GABA production was observed in 5 brix STE with 3% yeast extract. However, there is no information at the fermentation time for the highest GABA conversion over the period of fermentation. To investigate the effect of fermentation time on GABA production, *L. brevis* BJ20 was cultivated in 5 brix STE supplemented with 3% yeast extract. The GABA content was increased as the fermentation progressed, while the content of glutamic acid gradually decreased over the periods of fermentation and no determined after 108 h of fermentation. The highest GABA production was observed at 120 h of fermentation and about 50% of glutamic acid was converted into GABA (Fig. 26). In contrast to 5 brix STE containing 3% yeast extract, glutamate acid almost did not convert to GABA in Lactobacilli MRS broth supplemented with 3% yeast extract. In these conditions, the highest GABA content of 0.234% was observed (Fig. 27). Thus, *L. brevis* BJ20 is the most suitable bacterial strain to convert GABA in STE.

Di cagno et al., (2010) and Higuchi et al., (1997) reported that *L. plantarum* DSM19463 and *L. paracasei* NFRI7415 required 72 h and 144 h of fermentation to reach the highest production of GABA, respectively. According to other report, black raspberry juice was shown the highest

GABA production at the 15 day of the fermentation by *L. brevis* GABA 100 (Kim et al., 2009). It was thought that the different fermentation time to achieve the highest GABA production was also from the difference on bacterial strain, culture medium and others.

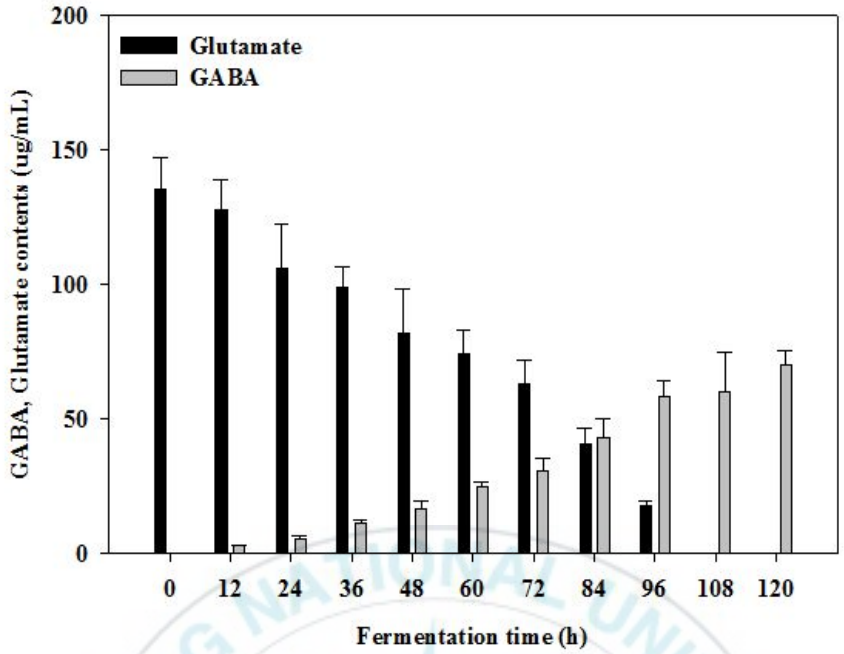

Fig. 26. Effect of fermentation times on GABA production by *Lactobacillus brevis* BJ20 in 5 brix sea tangle extract with 3% yeast extract.

Lactobacillus brevis BJ20 was fermented at 37°C with stationary. Data represent the mean values for three replicate experiments. Bars represent standard deviations (0.05<P).

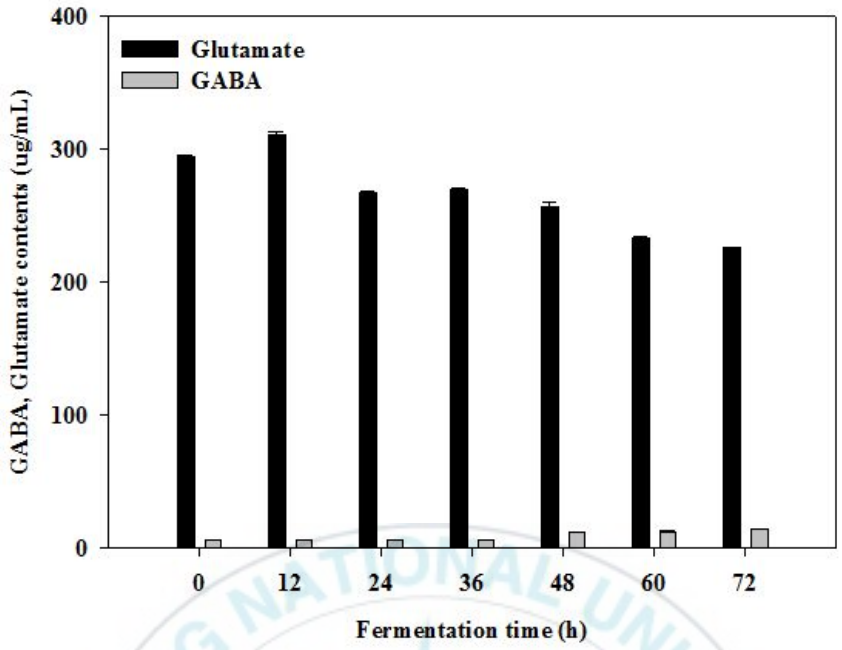

Fig. 27. Effect of fermentation times on GABA production by *Lactobacillus brevis* BJ20 in Lactobacilli MRS broth with 3% yeast extract.

L. brevis BJ20 was fermented at 37°C with stationary. Data represent the mean values for three replicate experiments. Bars represent standard deviations (0.05<P).

5. Regulation of mRNA expression of GAD related genes during the fermentation of *L. brevis* BJ20 in STE

It seems that the GABA conversion by *L. brevis* BJ20 in STE medium was mainly affected by the presence of yeast extract (Fig. 24 and Fig. 25). It has been previously reported GAD related genes (*gadR*, *gadC* and *gadB*) mediated with the conversion of glutamic acid into GABA in *L. brevis* strains (Shi and Li, 2011; Zhang et al., 2010; Hiraga et al., 2008; Kim et al., 2007; Park and Oh, 2007; Lucas and Lonvaud Funel, 2002). To elucidate the regulation mechanism of GABA conversion in STE, the mRNA expression of GAD related genes was monitored by RT-PCR as described in Materials and Methods. *L. brevis* BJ20 cells were grown under the different nutritional conditions and then the mRNA expression of each gene was investigated.

As shown in Fig. 28, the GAD related genes, a key factor of GABA conversion, were only transcribed into mRNA in the condition of 5 brix medium supplemented with 3% yeast extract. Small and Waterman (1998) reported that glutamate transcriptional regulator encoded by *gadR* gene, gamma aminobutyric antiporter encoded by *gadC* gene and glutamate decarboxylase encoded by *gadB* gene were essential proteins involved in GABA conversion in cell and transport into the outside of cell. In addition,

these results were consistent with the results of GABA production that was affected by a nutrient composition in medium. Thus, the highest GABA conversion was observed in 5 brix STE with 3% yeast extract and also the mRNA transcription of GAD related genes was observed. These results indicate that the nutrient yeast extract will be an activator to trigger the conversion of glutamic acid during the fermentation in STE medium. However, no mRNA transcription was observed in the other nutrient combination mode (Lactobacilli MRS broth, 5 brix STE and 5 brix STE with 1% glucose), while the control GAPDH gene was clearly detected in all samples. Indeed, the addition of glucose into 5 brix medium supplemented with 3% yeast extract resulted in no mRNA transcription of GAD related genes, indicating that glucose will act as a repressor on the expression of GAD related genes in *L. brevis* BJ20 cell.

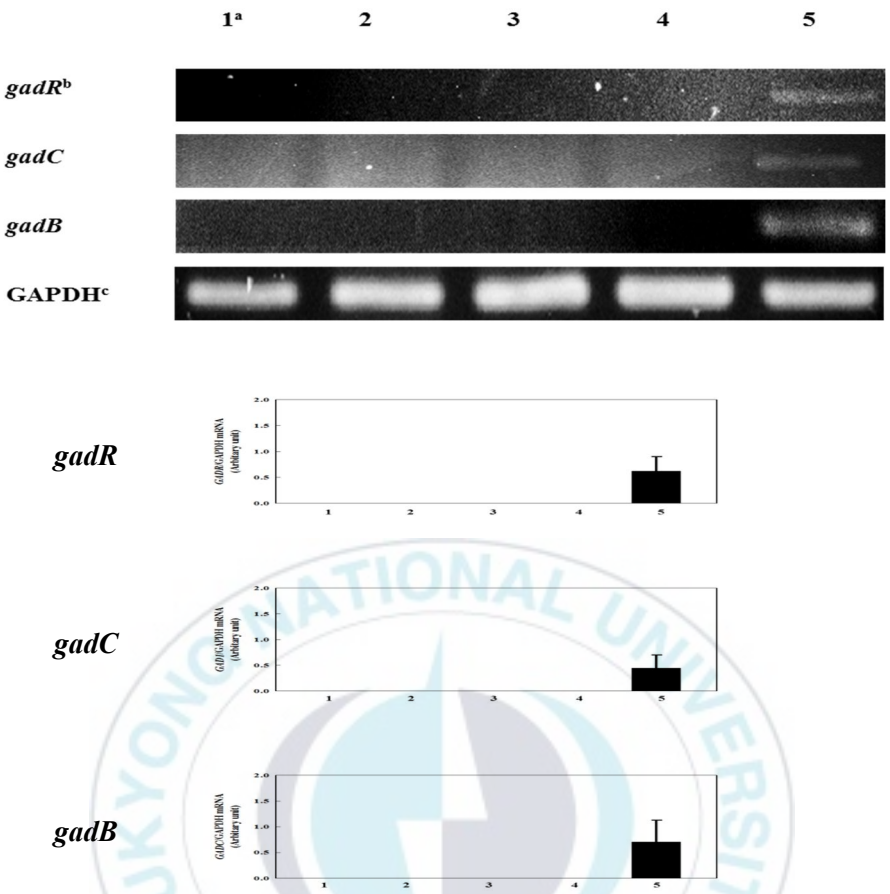

Fig. 28. Effect of yeast extract on mRNA expression of GAD related genes (*gadR*, *gadC* and *gadB*) in *Lactobacillus brevis* BJ20.

[a]1: 5 brix STE, 2: MRS broth, 3: 5 brix STE with 3% yeast extract and 1% glucose, 4: 5 brix STE with 1% glucose, 5: 5 brix STE with 3% yeast extract

[b]*gadR*: glutamate transcription regulator gene, *gadC*: gamma aminobutyric antiporter gene, *gadB*: glutamate decarboxylase gene, [c]GAPH: glycerldehyde 3-phsphate dehydrogenase gene.

Conclusion

This study was conducted to elucidate the mechanism of GABA production in STE fermented by *L. brevis* BJ20. The addition of carbon sources and amnion acids into STE did not affect on GABA production. GABA production was only observed in all STE media supplemented with nitrogen sources, even though the GABA production was affected by the content of nitrogen sources. Among of them, the highest GABA conversion was determined in 5 brix STE containing yeast extract. From these results, it was supposed that yeast extract will be is an essential factor on GABA production by the bacterial fermentation using *L. brevis* BJ20 in STE. The highest GABA production by *L. brevis* BJ20 was determined at the fermentation of 120 h in 5 brix STE supplemented with 3% yeast extract, while no GABA conversion was observed in Lactobacilli MRS broth supplemented with yeast extract. These results indicate that yeast extract will be an important factor in bioconversion of GABA by the fermentation of *L. brevis* BJ20 in STE. In order to address the mechanism of GABA conversion under these conditions, the mRNA expression of genes (*gadR*, *gadC*, *gadB*) associated with GABA conversion in *L. brevis* BJ20 cells was monitored by RT-PCR. The RT-PCR results revealed that the genes were

only transcribed into mRNA in the presence of condition 3% yeast extract in 5 brix STE medium. Considering above results, it was concluded that yeast extract will be a critical factor on the bioconversion of glutamic acid into GABA during the fermentation by *L. brevis* BJ20 in STE medium. Thus, the results obtained in this study will contribute to develop high value-added seaweed products that contain bioactive compounds such as GABA through fermentation.

Acknowledgement

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