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Full Length Research Paper

Microbial conversion of major ginsenoside Rb1 to minor ginsenoside Rd by Indian fermented food bacteria

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Ginsenoside Rb1 is the predominant secondary metabolite (saponin) in *Panax ginseng*. Hydrolysis of the sugar residues in Rb1 yields more pharmaceutically active ginsenosides like Rd, Rg3, F2, Rh2 and C-K. Among them, the minor ginsenoside Rd enhances the differentiation of neural stem cells, protects neurons from neurotoxic chemicals, decreases urea nitrogen and creatinine in kidney. It also protects the kidney from apoptosis and DNA fragmentation caused by cancer and chemical drugs and is more useful therapeutically than the major ginsenoside Rb1. Bacteria showing β- glucosidase activity were isolated from fermented Indian food using esculin-MRS agar. Bacteria from Amla in sugar syrup and Boiled Amla in jaggery syrup converted ginsenoside Rb1 to minor ginsenoside Rd. TLC and HPLC analysis showed that with increase in incubation time the conversion of Rb1 to Rd also increased. The 16s rDNA sequence was determined and the bacteria showed 93% sequence similarity to *Brumimicrobium mesophilum.*

Key words: Conversion, *ginseng,* ginsenoside, Rb1, Rd.

INTRODUCTION

Panax ginseng is one of the most popular medicinal plants known for its wide range of therapeutic activities. The roots of *ginseng* have traditionally been used for their nutritional and medicinal properties. They strengthen immunity and help in recovering health from fatigue. *Ginseng* roots contain various secondary metabolites-saponins, polyacetylenes, polyphenolic compounds and acidic polysaccharides. Among them *ginseng* saponins known as ginsenoside are the most important secondary metabolite and are the most pharmaceutically active. Until now 50 ginsenosides have been isolated with five major ones Rb1, Rb2, Rc, Re and Rg1 constituting more than 80% of the total ginsenosides (Kim et. al., 1987).

In recent times, many studies have been focused on the pharmaceutical activities of minor ginsenosides Rd, Rg3, Rh2 and compound K due to their superiority over the major ginsenosides. The minor ginsenosides are present only in small percentages and are usually the hydrolysis product of the major ginsenosides. Therefore, many studies have been aimed at converting major ginsenosides to the less available more active minor ginsenosides. The methods used for hydrolysis include heating, acid treatment and enzymatic conversion. Heating and acid treatment are not preferred as they modify the native structure and acidic polysaccharides by randomly hydrolyzing glycosidic bonds. These can remove the other pharmaceutical activities of *ginseng* root extracts.

However, enzymatic conversion is the most appropriate method and is desirable at a specific position for the production of particular active minor ginsenosides. Many studies for the production of the active minor ginsenosides Rd, Rg3, Rh2 and compound K have been carried out using both commercial and microbial enzymes (Kim et al., 2005; Cheng et al., 2006). The most abundant

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major ginsenoside Rb1 (23%) (Kim et al., 1987) can be easily converted to ginsenoside Rd by hydrolysis of one glucose residue.

There are various therapeutic activities of Rd apart from being an 'adaptogen' just like any other ginsenoside. The extraction of the major ginsenosides is much easy when compared to the minor ones and thus ginsenoside Rd can be produced from it. Rd, being a minor ginseno-side is found in low amounts in the natural habitat and hence can be converted from Rb1. Ginsenoside Rd is known to decrease levels of urea nitrogen and creatinine in kidney and has demonstrated protection against renal dysfunction caused by ischemia and recirculation (Yokozawa et.al., 1998). It protects kidney from apoptosis and DNA fragmentation caused by chemical drugs and cancer (Yokozawa and Owada, 1999; Yokozawa and Liu, 2000; Yokozawa and Doug, 2001).

Ginsenoside Rd is also known to help learning and memory functions in mice and prevent contraction of blood vessel; thereby preventing blocking of blood circulation (Zeng et al., 2003). The pharmaceutical property of ginseng roots in protecting neurons from neurotoxic chemicals is attributed mostly to ginsenoside Rd. It is known to protect neural systems against neuro toxicity by attenuating NO overproduction (Choi et al., 2003). It arrests the aging process of the suppressive anti oxidative defense system and lipid peroxidation by elevating the GSH/GSSG ratio of glutathione and increases activity of glutathione reductase and glutathione peroxidase which are significantly lower in older organisms. It enhances the differentiation of neural stem cells, while other ginsenosides induce no differentiation of neurons (Shi et al., 2005).

This study is aimed at isolating a bacterial source for β-glucosidase enzyme which can be used for the conversion of Rb1 to Rd. Earlier studies on bioconversion has used β-glucosidase producing bacteria from soil of *ginseng* field (Kim et al., 2005) and commercially available food grade micro organisms (Chi and Ji, 2005). Food grade micro organisms when ingested by humans do not cause any disturbances. Therefore, β-glucosidase producing bacteria was isolated from fermented Indian food as they are safe and no controversy regarding their use for bioconversion could arise.

MATERIALS AND METHODS

Selection of food source

Many fermented Indian food like Dosa batter, Dosa batter supplemented with rice flour, wheat, carrot, rava, rye flour, paneer whey and Amla (*Amlica embilicus)* in sugar or jaggery syrup were checked for β-glucosidase producing micro organisms. Amla was separated into 4 batches: boiled amla soaked in sugar syrup, in the second batch amla was soaked in sugar syrup without boiling stage, third - boiled amla soaked in jaggery syrup and fourth - soaked amla in jaggery syrup without boiling. All the four batches were left for fermentation for a 25 day period. These food sources were selected as they are consumed as fermented products, which will also favour growth of microorganisms. These microorganisms utilize the nutrients in the food source for their growth and survival and may produce beneficial enzymes.

Isolation of bacteria producing β-glucosidase and its growth characteristics

From the mentioned sources, sample was plated on MRS agar; the morphologically different colonies were identified and individually plated, tested for their β-glucosidase production on Esculin - MRS agar. The Esculin positive colonies produced dark brown zones on incubation for a period of 2 days at 37°C. The one bacterial culture from each of the two food sources - raw amla in sugar syrup named as A_rS and boiled amla in jaggery syrup- A_bJ were selected for the present study.

Assay of ginsenoside Rb1 converting activity of the β-glucosidase producing bacteria:

The bacterial cultures were grown for 2 days in MRS broth at 37°C. Prepared the reaction mixture with 200 µl of the above grown pure culture and 200 µl of major ginsenoside Rb1. The reaction mixture was incubated at 37°C for 2 days and bioconversion activity was checked by TLC.

The cultures that showed significant conversion to ginsenoside Rd were taken and verified their bioconversion activity by growing them for a period of 4 days and the incubation time of the reaction mixture was varied to 2, 7 and 16 day period. The reaction mixture was then extracted twice with 200 µl of butanol for analysis.

TLC analysis

Thin layer chromatography was performed on Silica gel60 F_{254} plates (Merck). A solvent mixture of Chloroform: methanol: water (65: 35:10 v/v/v, lower phase) was used as the developing solvent. The spots were detected by spraying 10% sulphuric acid followed by heating under lamp flame till the spots were clearly visible.

HPLC analysis

The butanol extract of the reaction mixture was evaporated in a vacuum evaporator and was redissolved in 200 µl of 100% HPLC grade methanol. Filtered using PFTE membrane filter of diameter 13 mm with pore size of 0.2 µm (MFC Advantec AFS.Inc) and 25 µl of the sample was injected for the HPLC analysis (Manufacturing company: FUTECS.Co.Ltd).

The HPLC was performed using C-18 reverse phase column (SUPELCO), using acetonitrile and water as solvent A and B respectively at 1.6 ml/min flow rate and the detection was at 203 nm. The A/B ratios are 15:85, 15:85, 21:79, 45:55, 90:10, 90:10, 15:85, 15:85 with run times of 1, 5, 25, 65, 75, 85, 87, 100 min respectively.

Phylogenetic study- 16S rDNA sequencing

Single colonies of the cultures with bioconversion activity were grown on MRS agar and the 16S rDNA sequences were obtained from Genotech in Daejeon, South Korea. The universal primers used were 9F and 1512R and the full sequences of the 16S rDNA sequence was compiled using SeqMan software and edited using BioEdit program (Hall, 1999). The sequences were then subjected to NCBI- BLAST program and the near relatives of the bacteria were determined.

Table 1. Constituents of MRS agar medium with Esculin.

Content	Amount (g/L)
Protease peptone	10
Beef extract	10
Yeast extract	5
Dextrose	20
Polysorbate 80	1
Ammonium Citrate	2
Sodium Acetate	5
Magnesium sulfate	0.1
Manganese sulfate	0.05
Esculin	3
Ferric ammonium citrate	0.2
Agar	15

RESULTS

Screening and assay of Indian fermented food for bacteria producing β-glucosidase

Many Indian fermented food sources were screened for β-glucosidase producing bacteria along with Amla (boiled and raw) soaked in sugar or jaggery syrup for a period of 15 days. A small amount of the fermented sample was mixed in sterile water and plated on R2A agar medium as it facilitates bacterial growth. Many different colonies were obtained and the morphologically different colonies were identified and streaked on MRS agar, specific for the growth of Lactic acid bacteria to obtain pure cultures. The plates were incubated at 37°C for a period of 2 days and checked for bacterial growth.

The single colonies of the bacteria obtained were spotted on MRS agar containing esculin (3.0 g/l). The bacteria with β-glucosidase activity produced dark brown to black colored zones around the colony on the MRS+esculin medium (Table 1) . Four bacteria were identified as esculin positive and these were taken for studying their bioconversion activity.

TLC analysis

The β-glucosidase producing bacterial strains were assayed to verify their hydrolytic activity of converting major ginsenoside Rb1. The pure cultures of the 4 bacteria were grown in 5 ml MRS broth at 37°C for 2 days at 170 rpm. The reaction mixture was prepared by inoculating 200 µl of Rb1 (1000 ppm) into 200 µl of the bacterial culture and incubated the mixture at 37°C for 2 days at 170 - 190 rpm.

Butanol extraction was then performed by adding 200 µl of butanol: water (7:3), vortexing and centrifuging at 13,000 rpm at 4°C for 6 min. The butanol layer was collected and the extraction was repeated. The extracts were pooled and then subjected to TLC analysis. The saponin standard containing Rb1, Rd, Rg3, F2, Rh2 and C-K, each in 1000 ppm concentration and the 3 butanol extracts were spot on the plate and placed in the solvent mixture of Chloroform: methanol: water (65:35:10 v/v/v, lower phase), used as the developing solvent. The spots were detected by spraying 10% sulphuric acid and heated under the flame of lamp and the spots developed were analyzed.

All the 4 cultures converted major ginsenoside Rb1 to minor ginsenoside Rd. The food sources were raw amla in sugar syrup and boiled amla in jaggery and 2 bacterial cultures were obtained from each. In order to confirm the results obtained the bacterial growth was extended to a period of 4 days and the bioconversion activity was tested at different time periods of 2, 7 and 16 days after the inoculation of Rb1. It was observed that, with in-crease in incubation time period, the bioconversion activity increased, thereby confirming the results obtained.

HPLC analysis

HPLC analysis was performed to verify the conversion of ginsenoside Rb1 to minor ginsenoside Rd. Three of the four strains showing bioconversion activity in TLC analysis were selected for the HPLC analysis. The HPLC analysis was performed using C-18 reverse phase column. The butanol extract was evaporated using the vacuum evaporator and redissolved the residue in 200 µl of HPLC grade methanol and filtered using 0.2 µ PFTE membrane filter of 13 mm diameter, 25 µl of the sample was injected for the analysis. With the results obtained it was confirmed that the 3 selected cultures converted Rb1 to minor ginsenoside Rd (Tables 1 and 2, Figures 1 and 2)

DISCUSSION

Ginsenoside Rb1 can be converted to the minor ginsenoside Rd by simple hydrolysis and loss of a glucose moiety from the C-20 position of ginsenoside aglycone. Many enzymes hydrolyzing the glycosidic bonds are known, of which -glucosidase is regarded as the most useful (Bae et al., 2000; Park et al., 2001). In the present study, attempts were made to screen many active -glucosidase- producing bacteria. Earlier bacteria have been isolated from soil around the roots from a ginseng field (Kim et al., 2005).

Also, reports on β-glucosidase producing human intestinal bacteria converting the major ginsenoside Rb1 to ginsenoside Rd are available (Bae et al., 2000). Since aerobic bacteria grow faster and produce enzymes in greater quantities than human intestinal bacteria and fungi, aerobic bacteria can be more effectively used for large scale enzyme preparation (Coskun and Ondul, 2004;

Table 2. HPLC conditions.

Total time	Time	Acetonitrile (100%)	Water (100%)
0	0	15	85
5	5	15	85
25	20	21	79
65	40	45	55
75	10	90	10
85	10	90	10
87	2	15	85
100	23	15	85

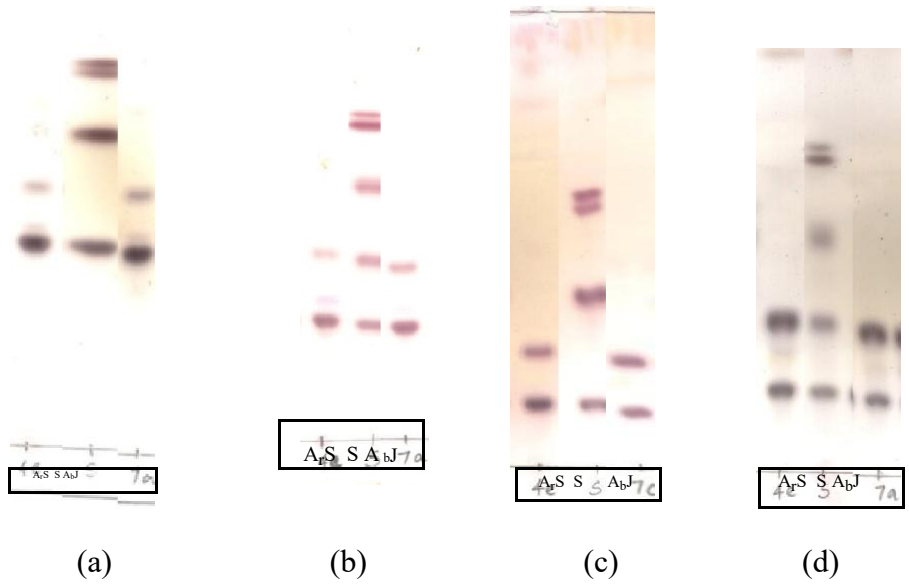

Figure 1. TLC chromatograms of the Ginsenoside Rb1 metabolites formed by β-glucosidase producing strains from the fermented food source amla. The major Ginsenoside Rb1 is converted to Minor Ginsenoside Rd by both the bacteria. The amount of conversion increases wit the increase in time period. (a) TLC result of 2 days growth and two days incubation with Rb1. (b) 4 days growth and 2 days incubation with Rb1. (c) 4 days growth and 1 week incubation with Rb1. (d) 4 days growth and 16 days incubation with Rb1.

Yoon et al., 2004) . The present study is focused on isolation of aerobic bacteria from Indian fermented food as their use for bioconversion will be safe and possibly no contradictory issues would arise.

The medium MRS agar is used for the isolation of bacteria since it specifically facilitates the growth of *Lactobacillus* and related species. The Esculin-MRS agar is specially designed to screen for -glucosidase producing bacteria. Esculin was needed to detect the -glucosidase activity, whereas MRS was required to support bacterial growth (Kim et al., 2005).

After 48 h of growth, the bacterial suspension was treated with equal volume of Rb1 (1000 ppm) and the bioconversion was checked by TLC after a 2- day incubation period. As no remarkable conversion was observed, the growth period was extended to 4 days and the bioconversion was studied at different time periods of 2 days, 1 week and 16 days.

During the 2 day growth period not much of the enzyme seemed to be produced as there is not much increase in Rd. Therefore, the growth time was increased to a 4 day period so that there may be an increased production of the enzyme -glucosidase. From the results obtained it is clear that with increase in incubation time the bioconversion also increased, observed by the disappearance of Rb1 and better intensity of the Rd band. Kim et al. (2005) observed that a strain GP50 on longer incubation time with Rb1 produced compound K. Anticipating similar results, the cultures A_r S and A_bJ were kept for a longer incubation time of one week and 16 days. However, only conversion to Rd was found to increase as deduced by the increase in the intensity of the spots on the TLC plate.

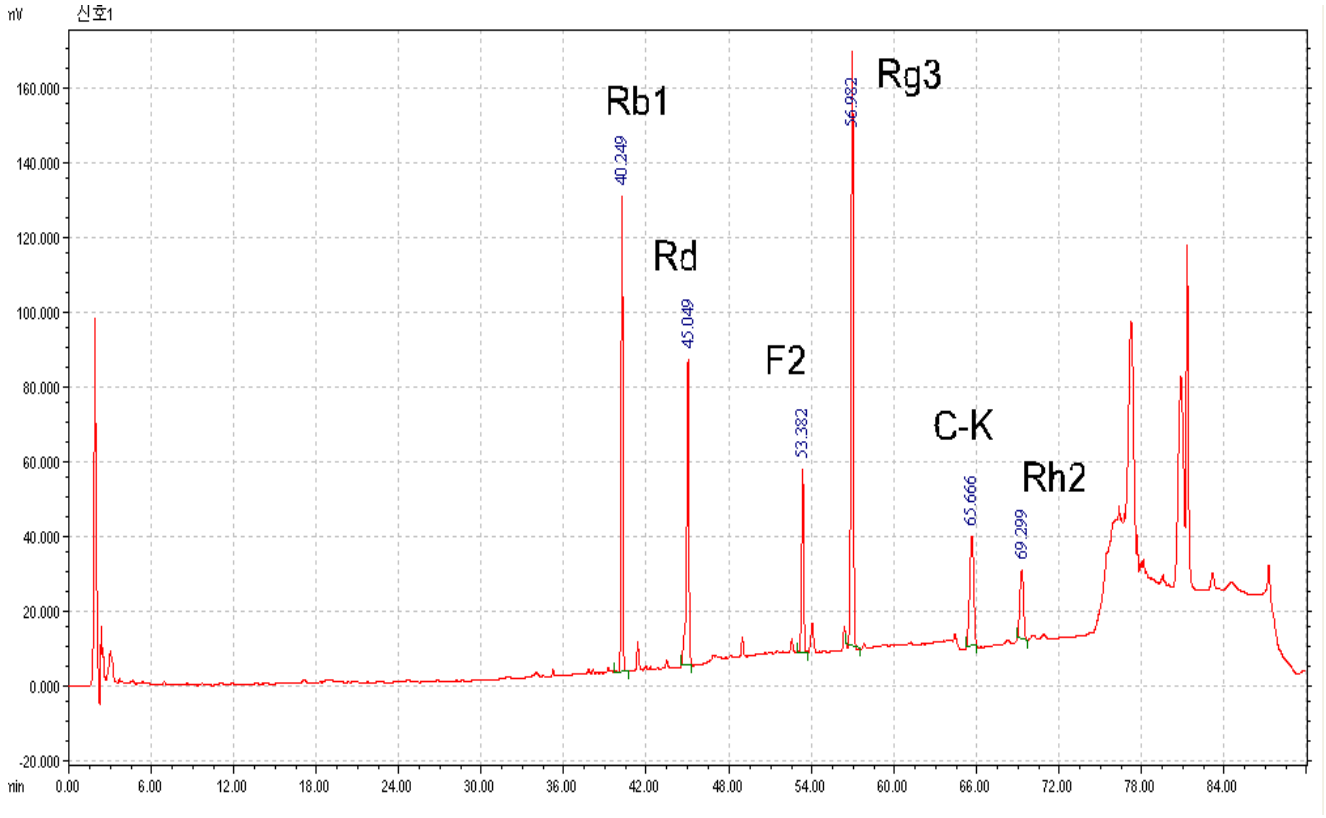

Figure 2a. Standard ginsenoside HPLC chromatogram.

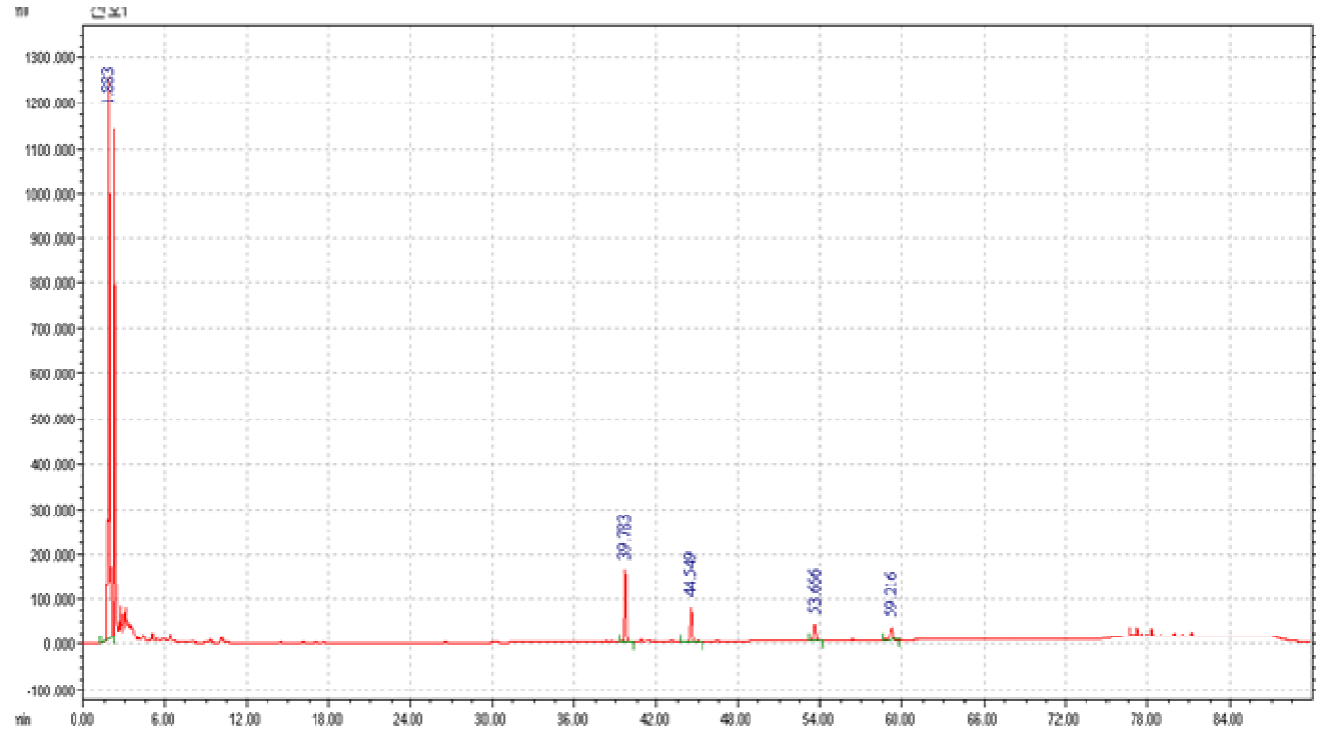

Figure 2b. Culture A_rS- 1 week incubation.

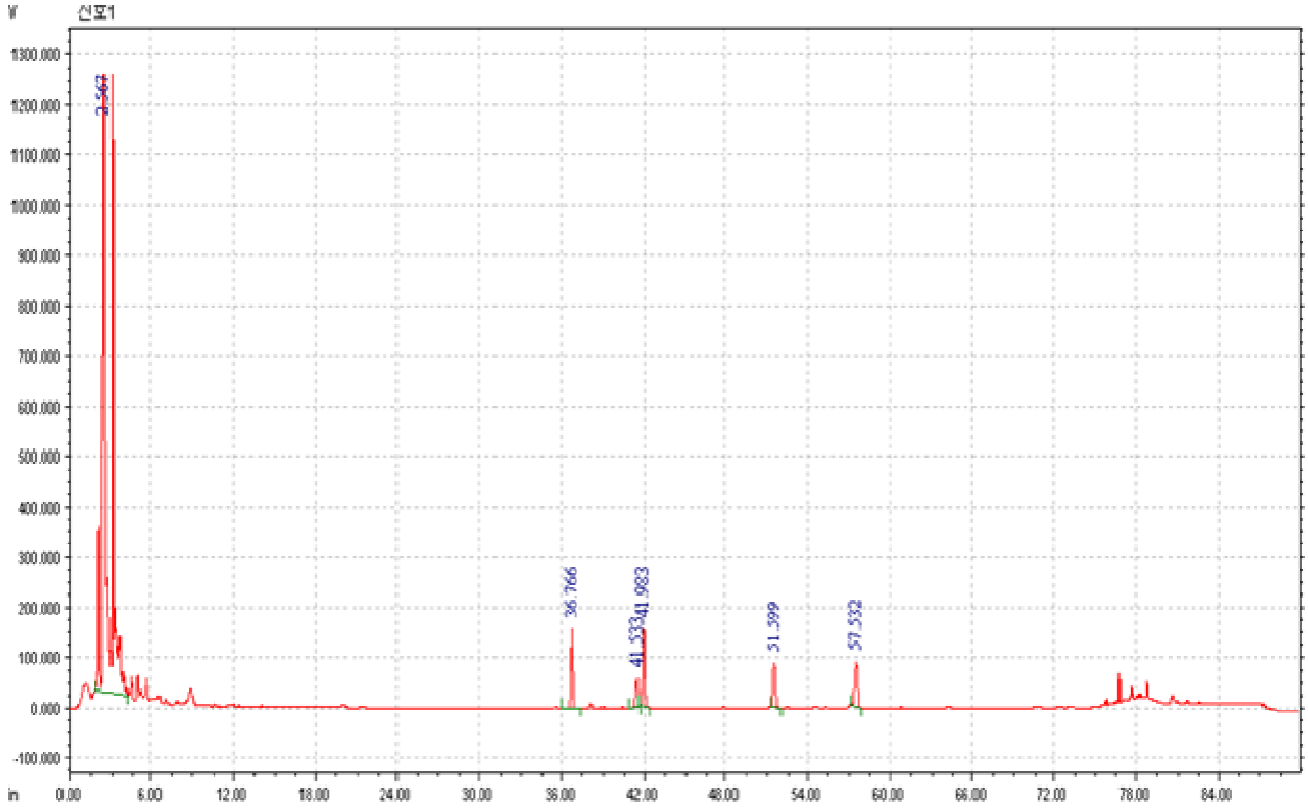

Figure 2c. CultureA_bJ-1 week incubation.

In the HPLC analysis, as already noted, the major ginsenoside Rb1 was converted to Rd. There is also some insignificant amount of conversion to the minor ginsenoside F2, but these minor peaks were not detected as spots in TLC analysis. Therefore, the substrate specificity of the enzyme needs further analysis. No evidence exists that the -glucosidases produced from the isolated bacteria play a significant role in the conver-sion of ginsenoside Rb1, as other enzymes secreted from the -glucosidase-producing microorganisms may also hydrolyze ginsenoside Rb1. Purification of the ginseno-side Rb1-converting enzyme from the isolated bacteria would verify the enzymatic reaction.

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