Kor. J. Microbiol. Biotechnol.
Vol. 38, No. 2, 129–135 (2010)

Production of Bioactive Compounds from Fungi Grown on Ginseng-Steaming Effluent

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We described production of bioactive compounds from fungi grown on Korean ginseng-steaming effluents (GSE) for develop high-value added nutraceuticals from Korean GSE. *Hansenula anomala* KCCM 11473, which grew well in Korean GSE had high RNA content, and its optimal autolysis conditions were established to produce 5'-ribonucleotides (13.9~28.5 mg/g of biomass) at 55°C and pH 5.0 for 24 h. 5'-Phosphodiesterase and adenyl deaminase were not effective in increasing the yield of 5'-ribinucleatides, but the yield of IMP increased significantly only after the addition of 1.0% adenyl deaminase. *Saccharomyces cerevisiae* showed the highest growth in the GSE medium. 267.1 mg of *S. cerevisiae* biomass was produced from 1 g of GSE solid and medicinal ginsenoside-Rg_3 contents was determined with 0.033 mg. *Mucor miehei* KCTC 6011 produced approximately 120 mg of chitosan per g-dry mycelium in 84 h at 25°C when grown in the GSE (pH 8.0) supplemented with 0.5% yeast extract and 0.002% $CuSO_4$. Chitosan produced by *M. miehei* KCTC 6011 have deacetylated approximately 56% and its viscosity and molecular weight of the chitosan were 80 cps and 1.07 x 10^3 kDa, respectively. The chitosan at 1.5 mg/ml inhibited 73.9% of the mycelium growth of *Rhizotonia solani* in 60 h.

Key words: Korean ginseng-steaming effluents (GSE), bioactive compounds, yeasts, filamentous fungi.

Introduction

Various ginseng products including ginseng tea and drinks are produced from ginseng extracts by ginseng processing companies. A large amount of by-products such as ginseng-steaming effluents (GSE) and red ginseng lees are discharged during the manufacturing process of ginseng extracts [13]. GSE contains high concentration of useful substrates [13]. However, only a small portion of GSE is recently utilized to extract useful ginsenosides or to produce maltooligosaccharides [12, 13] and mononucleotides [8] as agents for aroma therapy cosmetics, medical and functional foods, etc. Therefore, it is imperation to improve efficiency and develop high value-added nutraceuticals from GSE and further, to prevent environment pollution by GSE.

Fungi have been used to produce several bioactive compounds. Especially, yeast generally offers some industrial advantages, including rapid growth, ease of cultivation, eukaryotes, and the capacity to be grown in a cheap medium containing agricultural by-products [20]. Various yeasts have been used for the production of high-value bioingredients in food and animal feed, as well as for medicinal purposes with respect to ribonucleotides, industrial enzymes, and vitamins [9, 20]. Recently, bioactive compounds such as an antihypertensive angiotensin I-converting enzyme inhibitor [10], an antiangiogenic compound [7], anti-dementia β-secretase inhibitor [18] and ginsenoside-Rg_3 [15] were produced and characterized form *Saccharomyces cerevisiae*. Filamentous fungi (mold) has been also used to produce industrial enzymes, organic acids and enhancer of flavors and tastes for food industry. Recently, it is very useful to produce functional substances such as chitosan.

We now describe the bioactive compounds from fungi grown on Korean GSE for the efficient utilization of GSE and further for the prevention of environmental pollution by GSE.

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Production of ribonucleotides

5'-Ribonucleotides such as IMP and GMP have been used as flavor-enhancers in various foods. GMP shows strong flavor enhancement activity [2], and IMP has also lower moisture absorption, which is advantageous in dry mixed products and flavors [23, 25]. In addition to their use in the food industry, ribonucleotides, nucleosides, and their derivatives have been shown to exhibit various therapeutic and immunostimulatory effects, and thus, are used in animal feed, human supplements, as well as medical ingredients [2]. Supplements of purines and pyrimidines from RNA-rich yeast extracts promote protection of products against bacterial contaminations and stimulate immune functions [2]. One nucleotide derivative, 9-β-D-arabinofuranosyladenine, was also effective against *Herpes simplex* and *Herpes zoster* in humans [3].

Various microbial sources such as *Candida utilis*, *Penicillium citrinum*, *S. cerevisiae*, *Kluyveromyces marxianus*, *Brevibacterium ammoniagenes*, *Streptomyces aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Micrococcus glutamicus*, *Methylobacter acidophilus*, and *Escherichia coli* have been reported to produce ribonucleotides and their derivatives, which are high-value bio-ingredients for food, feed and medical industries [4]. However, their RNA yields were generally low and costly to produce. *Hansenula anomala* KCCM 11473 showed the best growth and the highest RNA content in GSE without supplement with other nutrients [8] (Table 1). *Rhodotonula glutinis* S-1, *Saccharomyces pastorianus* KCTC 1218, and *Hansenula anomala* KCTC 7295 also grew well in the GSE. RNA content of *H. anomala* KCCM 11473 increased with increasing cell growth, and the maximum RNA content (3.4 ug/ml of cultures) was obtained in the mid-log phase of growth, but eventually decreased with cultivation time. These results were similar to those for *Sacchromyces cerevisiae* FL 521 [26] and *Sacchromyces cerevisiae* ATCC 7754 [15], which are considered to be due to the inhibition of RNA synthesis by deficiency of nutrients such as glucose and amino acid [27]. Most of the sugars in GSE were consumed in 6 h of fermentation by *Hansenula anomala*. Thus, fermentation by *H. anomala* to produce ribonucleotides would be an effective process to reduce disposal problems of the GSE. The yield of 5'-rinonucleotides in the autolysates were varied from 0.02 mg (IMP) to 22.9 mg (AMP) per gram of biomass (dry weight). The yield of 5'-ribonucleotides was strongly influenced by autolysis temperature and pH, and the highest levels of AMP and IMP were produced within 6 h at 55°C, pH 5.5, while the GMP level was maximal at 6 h at 55°C, pH 5.0. These results are different from those for *K. maxianus* grown on whey (50°C, pH 6.5) [2]. The autolysis duration slightly influenced the yield of 5'- ribonucleotides. After 24 h of autolysis, the yield of 5'-ribonucleotides increased to about 3.0-5.0 mg/g of biomass as compared with that at 6 h. Belem *et al.* [2] reported that after 12 h autolysis of *K. marxianus, the* yield of 5'-IMP was about three times higher than that of after 6 h.

To increase the specificity of RNA hydrolysis and the rate of conversion of 5'-ribonucleotides, enzymatic treatment with 5'-phosphodiesterase and adenyl deaminase was performed. The effect of 5'-phosphodiesterase on the autolysates was not significant. By increasing the concentration of 5'-phosphodiesterase, the yield of 5'-ribonucleotides decreased, which was not in agreement with the results for *K. maxianus* [2]. The IMP yield increased to 20.4 mg/g of biomass (d.w.) after the addition of 1.0% adenyl deaminase. However, the effect of adenyl deaminase on GMP was not

Table 1. Growth and RNA yields of various yeasts in Korean ginseng steaming effluent [8].

Yeast	Growth (A_{660})	RNA yield (mg/ml-culture)
Candida edax OE-17	0.87	0.42
Rhodotonula glutinis OE-18	0.79	0.31
Candida spp. (group III) OE-23	0.08	-
Candida spp. (group III) OE-25	0.37	0.20
Rhodotonula glutinis S-1	1.32	0.49
Saccharomyces spp. S-3	0.49	0.41
Kluyveromyces spp. S-7	0.23	0.54
Candida incommunis S-8	0.33	0.88
Hansenula spp. S-9	0.25	0.57
Zygosaccharomyces rouxii S-10	0.36	0.76
Saccharomyces pastorianus KCTC 1218	1.11	0.99
Saccharomyces cerevisiae T-71	0.32	1.04
Saccharomyces cerevisiae HG-7	0.63	0.73
Saccharomyces cerevisiae HG-10	0.56	0.99
Zygosaccharomyces rouxii KCCM 12066	0.15	-
Zygosaccharomyces mellis KCCM 50160	0.14	-
Candida tropicalis KCTC 7725	0.14	-
Candida krusei KCTC 7213	0.14	-
Candida versatilis KCTC 7220	0.14	-
Kluyveromyces lactis KCTC 7138	0.40	0.22
Kluyveromyces fragilis KCTC 7260	0.13	-
Pichia membranaefaciens KCTC 7006	0.50	
Hansenula anomala KCTC 7295	1.14	2.15
Hansenula anomala KCCM 11473	1.49	2.77

apparent and instead the AMP yield decreased by treatment of adenyl deaminase. The final yield of AMP, GMP, and IMP were 28.6, 12.6 and 20.4 mg/g of biomass (d.w.) after autolysis and enzymatic treatments, respectively. The yield of 5'-ribonucleotides from *H. anomala* autolysate was higher than those from extracts of *S. cerevisiae, K. maxianus,* and brewery yeast [2].

To increase yield of 5'-ribonucleotides from *Pichia anomala* (ex- *H. anomala*), various mutants of *P. anomala* were isolated by ethyl methanesulfonate (EMS) treatment and UV irradiation through cycloheximide resistance and KCl sensitivity [19]. The HA-2 positive mutant showed resistance to cycloheximide (300 ppm), 1.5 mol/L KCl, and its RNA content was about 35% higher when compared with the wild-type strain. HA-2 mutant also exhibited superior growth characteristics [8, 19].

The RNA content of *Pichia anomala* HA-2 mutant increased with the cell growth, and the maximum RNA content (55 mg/g dry wt.) was obtained at the late-log phase of growth, but eventually decreased after 12 h period of cultivation, which are considered to be due to the inhibition of RNA synthesis by deficiency of nutrients such as glucose. Similar characteristics have been observed from wild-type strain *P. anomala* [8]. The maximum yield of AMP (7.8 mg/g biomass) from the mutant, HA-2 was obtained at 60°C, pH 6.5, while GMP and IMP were maximal at 60°C, pH 7.0, both treated after 6 h of autolysis. These results are quite different from those obtained with wild-type *P. anomala* (55°C, pH 5.5) [8] and *K. maxianus* (50°C, pH 6.5) [2]. The maximum yield was obtained after 6 h of autolysis period, but eventually decreased with autolysis period.

Autolysates of *P. anomala* HA-2 contained large amounts of proteins or peptides. Consequently, some related physiological activities were evaluated [14]. Antihypertensive angiotensin I-converting enzyme (ACE) inhibitory activity and nitrite-scavenging activity of the mutant autolysates were 16.0% and 47.0% respectively, higher than the native strain. Although the ACE inhibitory activity of *P. anomala* HA-2 was relatively low when compared with other sources, it may be useful as a potential new source of antihypertensive compounds. Furthermore, the high nitrite-scavenging activity of mutant HA-2 shows some promise for the development of functional health foods in the therapy or prevention of nitrite-related cancers and other diseases. From above results, the technology developed may be useful to produce ribonucleotide-rich yeast extract or bioactive compounds from these yeasts for potential food application as well as for solving waste the disposal problem regarding Korean GSE.

Production of medicinal ginsenoside-Rg3 enriched yeast

Generally, ginsenoside-Rg_3 is produced during processing of ginseng and also converted from ginsenoside-Rb_1, Rb_2 and Rc by treatment of slight acid and heat (100°C, 20 min) or bioconversion of the microbes during cultivation [14]. It is known that ginsenoside-Rg_3 have anti-cancer by inhibition of AP-1 and JNK activity, anti-dementia by β-secretase (production of β-amyloid protein) inhibition, coronary artery contraction, anti-stress by reduction of putrecine, brain protection by improvement of brain blood circulation and anti-nerve denaturation by glutamate, etc [14]. We describe production of medicinal ginsenoside-Rg_3 enriched yeasts using GSE [15].

Growth of *Sacchromyces cerevisiae* KCTC 7904 and *Sacchromyces pastorianus* KCTC 7919 in the GSE were 276.1 mg per g GSE (1.33, A_{660}) and 226.5 mg (1.24, A_{660}). These results were higher than those of other yeasts

Table 2. Effect of autolysis temperature and pH on yield of 5'-ribonucleotides from *Hansenula anomala* KCCM 11473 [8].

Temp. (°C)	pH	Yield (mg/g of biomass)[1)]		
		5'-AMP	5'-GMP	5'-IMP
45	6.0	0.04	0.04	0.02
	6.5	0.08	0.07	0.3
	7.0	0.1	0.2	1.1
	7.5	1.2	0.8	1.3
50	6.0	14.5	6.5	11.4
	6.5	12.1	5.2	10.4
	7.0	10.2	5.2	8.2
	7.5	5.1	3.3	4.6
55	5.0	20.9	9.8	14.5
	5.5	22.9	7.3	16.0
	6.0	17.1	6.9	13.3
	6.5	15.4	6.6	9.0
	7.0	8.0	6.1	6.3
	7.5	0.5	0.4	0.4
60	6.0	15.0	8.1	10.0
	6.5	10.0	4.8	7.7
	7.0	10.1	2.5	7.7
	7.5	0.8	0.2	0.7

[1)]After 6 h autolysis with duplicate analysis.

Table 3. Growth of various yeasts in Korean ginseng steaming effluent [15].

Yeasts	Growth	
	A_{660}	Weight (mg)[1)]
Saccharomyces cerevisiae KCTC 7904	1.33	276.1
Zygosaccharomyces rouxii KCTC 12066	1.02	166.2
Saccharomyces pastorianus KCTC 7919	1.24	226.5
Kluyveromyces flagilis KCTC 7260	1.12	181.4
Lipomyces starkeyi	0.52	86.1

[1)]Amounts of freeze dried biomass cultured form 1g of GSE solid.

grown on GSE (Table 3) [14]. The Ginsenoside-Rg_3 was identified in HPLC chromatogram of cell-free extract from *S. cerevisiae* KCTC 7904 grown on GSE and the other ginsenosides were not detected [14]. Compared with 9.66 mg/g GSE of ginsenoside-Rg_3 was contained in GSE itself, *S. cerevisiae* KCTC 7904 grown on GSE (1 g) contained 0.033 mg/g GSE of ginsenoside-Rg_3. Any ginsenosides were not detected in cell-free extracts from the yeast grown on YEPD medium (Table 4).

Production of chitosan

Mucor miehei KCTC 6011 showed the most satisfactory growth in the GSE without any added of nutrients and it produced maximally 0.4 mg of chitosan per g-dry mycelium after 3 days of culture at 25°C (Table 5) [11]. While *Rhizopus* species showed poor growth in the GSE, *Aspergillus* species grew very well in the GSE without producing chitosan.

Bartnicki-Garcia *et al*. [1] first reported the presence of significant quantities of chitosan in *Mucor rouxii*. Since it is known that *Mucorales* contained a high content of chitin in the cell walls, the production of microbial chitosan might depend on the cell wall composition [16]. It has been reported that some fungi such as *Absidia atrospora* IFO 09471, *Absidia coerulea* IFO 4011, *Absidia glauca* IFO 4002, *Absidia glauca* IFO 4003, *Absidia glauca* var. *paradoxa* IFO 4431, *Gongronella butleri* IFO 8080 and *Rhizopus japonicus* produced chitosan [5, 17]. Indeed, *Gongronella butleri* IFO 8081 produced 730 mg of chitosan/L after a 5- day culture in the sweet potato-shochu medium [6].

Table 4. Ginsenosides contents of the biomass from yeasts cultivated in Korean ginseng steaming effluent [15].

		Ginsenosides(mg/g solid)									
		Rb_1	Rb_2	Rc	Rd	Re	Rf	Rg_1	Rg_3	Rh_2	Total
GSE[1)]		29.91	15.19	16.78	7.99	4.68	2.28	2.25	9.66	1.92	90.66
YEPD	*Saccharomyces cerevisiae* KCTC 7904	-	-	-	-	-	-	-	-	-	-
GSE	*Saccharomyces cerevisiae* KCTC 7904	-	-	-	-	-	-	-	0.033	-	0.033
	Saccharomyces pastorianus KCTC 7919	-	-	-	-	-	-	-	0.014	-	0.014
	Kluyveromyces flagilis KCTC 7260	-	-	-	-	-	-	-	0.019	-	0.019
	Zygosaccharomyces rouxii KCTC 12066	-	-	-	-	-	-	-	0.017	-	0.017
	Lipomyces starkeyi	-	-	-	-	-	-	-	0.013	-	0.013

[1)]Ginseng steaming effluent.

Table 5. Growth and chitosan productivity of various fungi in ginseng-steaming effluent medium [11].

Strains [1)]	Dry mass of mycelium (g/L)	Chitosan productivity (mg/g-dry mycelium)
Rhizopus japonicus KCTC 6945	0.1	- [2)]
Rhizopus formosaensis KCTC 6947	0.1	-
Rhizopus nigricans KCTC 6062	0.1	-
Mucor miehei KCTC 6011	0.6	0.4
Mucor ambiguus KCTC 6142	0.3	0.2
Mucor racemosus KCTC 6119	0.3	0.1
Aspergillus niger	0.4	-
Aspergillus nidulance	0.4	-

[1)]The strains were cultivated in GSE medium at 25°C for 3 days
[2)]-; < 0.1 mg/g-dry mycelium.

A considerable amount of chitosan (57.2 mg of chitosan/g-dry mycelium) from *M. miehei* KCTC 6011 was produced by adding the yeast extract (0.5%) into the GSE and tryptone with peptone were also good sources of nitrogen for the production of chitosan. However, urea was not utilized and adding of inorganic nitrogen sources did not affect the production of chitosan at all. 79 mg of chitosan/g-dry mycelium was produced by adding 0.002% $CuSO_4$ into GSE and maximally 120 mg of chitosan/g-dry mycelium was produced from *Mucor miehei* when the initial pH of the GSE was adjusted to 8.0 and cultured for 84 h at 25°C. The amount of chitosan that was produced by *M. meihei* in the GSE was higher than those of *Rhizopus japonicus*, *Rhizopus acetoinus* [24], *Aspergillus awamori* [22] and *Gongronella butler* [24].

Furthermore, 92% of COD_{Mn}, 89% of the total the sugar, and 94% of crude protein in the GSE were reduced by 84 h of cultivation of *M. miehei* KCTC 6011, together with chitosan production. Therefore, it can be said that this process could be used to prevent pollution. Chitosan from *M. miehei* KCTC 6011 was approximately 56% deacetylation degree, and its water-holding capacity and viscosity were 0.8 ml-H_2O/g-dry chitosan and 80 cps, respectively. Molecular weight of the chitosan was 1.07×10^3 KDa and the chitosan inhibited mycelium growth of *Rhizotonia solani* approximately 74% at 1.5 mg/mL of the chitosan (Table 6). Several studies on antifungal activity of chitosan have been published [5, 21] and Lee *et al.* [21] reported that more than 90% of the growth of *Botryosphaeria dothidea* were inhibited at 1.0 mg/mL of commercial chitosan, and Yun *et al.* [28] also reported that growth of *Fusarium culmorom*, a plant pathogenic fungus was significantly inhibited by 0.025% of chitosan for 8 days of culture.

Overall, these results suggest that utilization of *M. miehei* grown in the GSE may be useful in the production of chitosan, thereby eliminating pollution problem as well as in inhibiting plant pathogens.

Conclusion

During the ginseng extracts manufacturing processes, a large amounts of ginseng-steaming effluents (GSE) are produced as side products. Since GSE contains various kinds of organic compounds, it may cause environmental pollutions if it is discharged without any pretreatments. Therefore, if we develop fermentation processes to produce useful products using GSE as a raw material, it will be useful in terms of recycling of waste materials as well as reduction of pollutant.

This review paper described the production of bioactive compounds such as nucleotides and medicinal ginsenoside-Rg_3 by yeasts using GSE. Chitosan production process by filamentous fungi grown on GSE was also described. These results suggest that GSE could be a useful raw material in production of nutraceuticals by fermentation process.

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Table 6. Effect of chitosan from *Mucor miehei* KCTC 6011 on the mycelial growth of some phytopathogenic fungi [11].

Concentration (mg/mL)	Inhibition rate (%)			
	Fusarium oxysporum FCU 428	*Fusarium solani* FSG 503	*Rhizoctonia solani* 920102	*Alternaria gaisen* CNU 5080
0.1	3.6	0	21.8	7.1
0.5	7.1	4.6	34.8	14.3
1.0	14.3	13.6	54.4	17.9
1.5	21.4	27.3	73.9	21.4

[1)]Strains were incubated for 60 h at 25°C on PDA media containing various concentrations of chitosan.

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(Received June 4, 2010/Accepted June 11, 2010)

국문초록

인삼 유출액에서 생육한 곰팡이로부터 생리 활성 물질의 생산

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본 논문은 한국인삼의 추출물 제조 시 부수적으로 생산되는 유출액에 의한 환경오염을 방지하고 나아가 이들로부터 고부가가치의 생리활성물질을 유출액에서 생육이 우수한 균류로부터 생산한 논문이다. 유출액에서 생육이 좋았던 *Hansenula anomala* KCCM 11473으로부터 5'-ribonucleotide 생산 최적 조건은 세포현탁액의 pH를 5.0으로 하고 55°C에서 24시간 자기소화 시키는 조건이다. 또한 생육이 좋았던 *Saccharomyces cerevisiae*는 유출액 배지에서 배양 중 약리 성분이 Ginsenoside-Rg3를 고형물 1 g당 0.033 mg을 생성하였다. *Mucor miehei* KCTC 6011을 유출액에 접종하여 25°C에서 84시간 배양했을 때 균체 건물 g당 120 mg의 키토산을 생성하였다.