

## Physiological Functionality and Enzyme Activity of Biomass from *Pichia anomala* Grown on Ginseng-Steaming Effluent

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A novel biomass was prepared from *Pichia anomala* KCCM 11473, which grew well in ginseng-steaming effluent (GSE), and its physiological functionalities and enzyme activities were determined. When the strain was cultured in the GSE (pH 6.0) at 30°C for 48 h, 1.6 mg of biomass per ml-cultures was produced. The cell-free extract of the biomass showed high antihypertensive angiotensin I-converting enzyme inhibitory activity of 72.0% and anticholesteromia HMG-CoA reductase inhibitory activity of 46.5%. The cell-free extract also showed 13.0 U per ml and 8.5 U per ml of neutral protease activity and alkaline protease, respectively.

**KEYWORDS :** Enzyme activity, Functionality, Ginseng-steaming effluents, *Pichia anomala*

Ginseng and its extracts have recently been used in the production of health foods, such as ginseng tea, ginseng drinks, and the extract itself. A large amount of ginseng steaming effluent (GSE) is discharged during the preparation of the ginseng extract at processing companies (Kim *et al.*, 2000). GSE contains useful substances, such as ginsenosides, sugars, and protein. However, only a small amount of GSE protein is used in the extraction of useful ginsenosides or in the production of maltooligosaccharides (Kim *et al.*, 2000) or bioactive compounds such as chitosan (Kim *et al.*, 2002) and ribonucleotides (Kim *et al.*, 2002). The bulk of GSE is discharged in the sewage, causing environmental pollution. It is imperative, therefore, to improve the efficiency of GSE utilization and to develop high value products from GSE.

Yeast generally offers some industrial advantages, including rapid growth, ease of cultivation, and the capacity to be grown in a cheap medium containing agricultural by-products (Kim *et al.*, 2004). Various yeasts have been used for the production of high-value bio-ingredients in food and animal feed, as well as for medicinal purposes with respect to ribonucleotides, industrial enzymes, and vitamins (Kim *et al.*, 2004). Recently, bioactive compounds such as an antihypertensive angiotensin I-converting enzyme (ACE) inhibitor (Kim *et al.*, 2004), an anti-angiogenic compound (Jeong *et al.*, 2006), and anti-dementia  $\beta$ -secretase inhibitor (Lee *et al.*, 2006) were produced and characterized from *Saccharomyces cerevisiae*.

In a previous study, we described the production of ribonucleotides by the autolysis of *Hansenula anomala* (Kim *et al.*, 2002), a *Pichia anomala* mutant (Lee *et al.*,

2004), and chitosan by *Mucor miehei* (Kim *et al.*, 2002) grown on ginseng-steaming effluent. We now describe the physiological functionality and some enzyme activity of biomass from *Pichia anomala* grown on ginseng-steaming effluent for the efficient utilization of ginseng-steaming effluent.

### Materials and Methods

**Strain, ginseng-steaming effluent (GSE), and chemicals.** *Pichia anomala* KCCM 11473, which was mentioned in a previous paper (Kim *et al.*, 2002) to have grown well in GSE, was used in this study. The GSE was obtained from a ginseng processing plant in Geumsan, in Chungnam province, South Korea. The GSE contained 64.0% total sugar, 34.2% crude protein, and 1.8% ash with 1125.4 mg/l COD<sub>Mn</sub>, and it had a pH of 4.95. The angiotensin I-converting enzyme (ACE) was extracted from rabbit lung acetone powder (Sigma Chemical Co., St. Louise, Mo., USA), and hippuric acid-histidine-leucine, fibrin, pyrogallol, and DPPH were also purchased from Sigma Chemical Co. (St. Louise, Mo., USA). Unless otherwise specified, all the chemicals were of analytical grade.

**Preparation of biomass.** *Pichia anomala* was inoculated in the GSE (pH 4.95) and cultured at 30°C for 72 h. After centrifugation of the culture broth at 10,000 ×g for 15 min, the yeast cells were harvested and subsequently dried with a vacuum freeze dryer.

**Assay of physiological functionality.** A cell-free extract of the biomass was prepared from the selected strain of

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yeast by cell disruption and centrifugation. We then used the following means to determine the cardiovascular and anti-aging functionalities. The antihypertensive ACE inhibitory activity was assayed by means of Cushman and Cheung's method (Cushman and Cheung, 1971). A mixture containing 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 3 units of ACE, and an appropriate amount of the cell-free extract was preincubated for 10 min at 37°C. The reaction was initiated by adding 50  $\mu$ l of Hip-His-Leu at a final concentration of 5 mM and terminated after 30 min of incubation by the addition of 250  $\mu$ l of 1.0 N HCl. The liberated hippuric acid was extracted with 1 ml of ethyl acetate, and 0.8 ml of the extract was dried with a Speed Vac Concentrator (EYELA Co., Japan). The residue was then dissolved in 1 ml of the sodium borate buffer. The absorbance at 228 nm was measured in order to estimate the ACE inhibitory activity.

The fibrinolytic activity was assayed by the method of Fayek *et al.* (1980) Each sample of 0.5 ml was added to 3 ml of a substrate solution (0.6% fibrin in 0.1 M McIlvaine buffer, pH 7.0) and incubated at 40°C for 10 min. The reaction was stopped by adding 3 ml of 0.4 M TCA for 30 min. The mixture was then filtered with Whatman filter paper No. 2. A reaction mixture of 1 ml of the filtrate, 5 ml of 0.4 M Na<sub>2</sub>CO<sub>3</sub>, and 1 ml of 1 N Folin reagent was placed at room temperature for 30 min. The amount of tyrosine released from the fibrin as a substrate was determined from the tyrosine standard curve by measuring the absorbance at 660 nm.

The HMG-CoA reductase inhibitory activity was assayed spectrophotometrically by measuring the rate of decrease in the absorbance at 340 nm due to the oxidation of NADPH (Kim *et al.*, 2005). A 0.5 ml volume of the reaction mixture contained the following: 50  $\mu$ M of a potassium phosphate buffer with a pH of 7.0, 2  $\mu$ M of dithiothreitol, NADPH, 0.3  $\mu$ M, 0.15  $\mu$ M of HMG-CoA, and 100  $\mu$ g of protein enzyme. Two reaction mixtures were preincubated in a 2 mm light path glass cuvette for 5 mins at 37°C. For the assay, we added HMG-CoA to one reaction mixture, and we added HMG-CoA with cell-free extract to the other reaction mixture. The mixtures were assayed at 37°C in a recording spectrophotometer. The initial velocity of the reaction was measured, and the net rate of the NADPH oxidation was determined by subtracting the rate of oxidation in the absence of HMG-CoA from the rate observed with both substrates present. Thus, we calculated the HMG-CoA reductase inhibitory activity as follows:

$$\text{HMG-CoA reductase inhibitory activity (\%)} \\ = \left\{ 1 - \frac{(A_{340} \text{ of sample} - A_{340} \text{ sample of blank})}{(A_{340} \text{ of control} - A_{340} \text{ sample of control})} \right\} \times 100$$

The nitrite-scavenging activity was assayed by the method of Kato *et al.* (1987) with NaNO<sub>2</sub> and Griess reagents.

The antioxidant activity was also assayed by the method of Blois (Blois, 1958) with 1,1-diphenyl-2-picryl hydrazyl (DPPH). A 0.8 ml DPPH solution (12.5 mg of DPPH dissolved in 100 ml of ethanol) was added to 0.2 ml of the sample, shaken for 10 s, and left for 10 mins. The absorbance at 525 nm was then determined and calculated as follows:

$$\left[ \frac{(A_{525} \text{ of reaction mixture} - A_{525} \text{ of sample alone})}{A_{525} \text{ of blank}} \right] \times 100.$$

The tyrosinase inhibitory activity was assayed by the method of Kim *et al.* (1997). The conversion of L-DOPA to a red-colored oxidation product, dopachrome, was measured spectrophotometrically. A 0.1 ml sample was incubated for 5 min at 30°C. At time zero, 1 ml of the L-DOPA (4 mg/ml) solution was rapidly added while stirring, and the absorbance was measured at 475 nm. After incubation for an additional 5 mins, the mixture was shaken again, and a second reading was determined.

$$\left[ 1 - \frac{(A_{475} \text{ of sample reaction mixture})}{A_{475} \text{ of blank}} \right] \times 100.$$

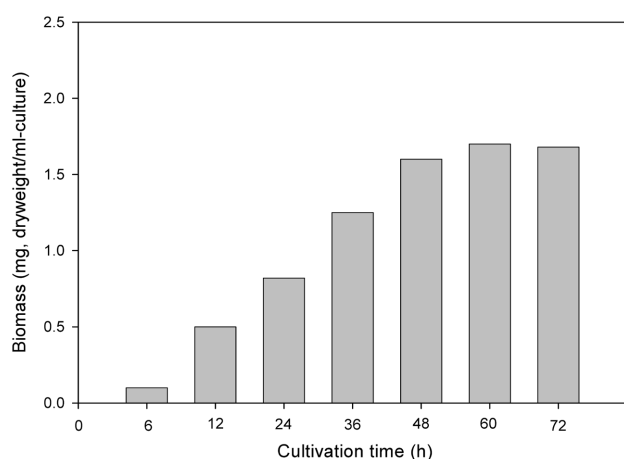
The glutathione S-transferase-activating activity of the cell-free extracts was measured by the method of Habig *et al.* (1974). The reaction system comprised 10  $\mu$ l of 1.0 mM 1-chloro-2,4-dinitrobenzene, 10  $\mu$ l glutathione, and 100  $\mu$ l of 0.1 mM phosphate buffer (pH 6.5). The mixture was preincubated for 5 min at 25°C. After the addition of the 10  $\mu$ l sample and 15  $\mu$ l of GST (1.5 unit) to the system, the change of absorbance at 314 nm for 5 min was measured. Distilled water was used as a blank, and the GST-activating activity was calculated as follows.

$$\text{GST-activating activity (\%)} \\ = \frac{(A_{314} \text{ of reactants} - A_{314} \text{ of sample itself})}{(A_{314} \text{ of control (blank)})} \times 100$$

**Assay of enzyme activity.** The activities of acidic, neutral, and alkaline proteases were spectrophotometrically determined by using different buffer solutions (pH 3.0, 7.0, 10.0) containing 0.6% skim milk and Folin reagents (Lee *et al.*, 1997).  $\alpha$ -Amylase and glucoamylase activity were determined using 1% soluble starch, and  $\beta$ -galactosidase and invertase activity were also determined using 4 mg/ml of ONPG and 2% sucrose as a substrate, respectively (Lee *et al.*, 1997). Inulinase activity was measured by determining the amount of the released reducing sugar from inulin (Kwon *et al.*, 1999).

## Results and Discussion

**Production of *Pichia anomala* biomass.** Fig. 1 shows the biomass content of *Pichia anomala* during fermentation. The biomass content increased as the cultural time



**Fig. 1.** Time course of biomass production from *Pichia anomala* KCCM 11473 in the ginseng-steaming effluent.

increased, and the maximum biomass content (1.6 mg/ml of cultures) was obtained at 48 h of cultivation.

**Physiological functionality of the biomass.** The functionality of the biomass was investigated (Table 1). The antihypertensive ACE inhibitory activity of the biomass was 72.0%, which is about 20% higher than that of the biomass from *Pichia anomala* grown on the yeast extract-peptone-dextrose (YEPD) medium containing 1% yeast extract, 2% peptone and 2% dextrose (51.2%). These results were also higher than those of the biomass from *Pichia anomala* (31.0%) and its mutant HA-2 (16.0%) grown on the yeast extract-malt extract (YM) medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% dextrose (Kim *et al.*, 2002), and the bio-

mass from *Saccharomyces cerevisiae* (42.1%) (Kim *et al.*, 2004). From reports on antihypertensive ACE inhibitory activity of ginseng extracts (Lee *et al.*, 2003) and adventitious root extracts of wild ginseng (Hong *et al.*, 2008), we guessed that ACE inhibitory activity of the biomass is caused by transference of antihypertensive agents in GSE into *Pichia anomala* during fermentation. ACE is known to regulate blood pressure by converting inactive decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin and inactivating the vasodilating nonapeptide bradykinin to raise blood pressure (Kim *et al.*, 2004). Recently, various ACE inhibitors with antihypertensive effects have been isolated via the enzymatic digestion of food protein, sake and its by-products, Korean traditional rice wines, cereals and legumes, and microbes such as yeast and mushrooms (Kim *et al.*, 2004).

To investigate industrial application for ACE inhibitor of the biomass, the ACE inhibitor of cell-free extract was partially purified by ultrafiltration and systematic solvent extraction. It was ultrafiltered with 5000Da cut-off filter (Labscale TFF System, Millipore Co., USA) and then ACE inhibitory activity of the filtrate was determined. Its ACE inhibitory activity was 61.6% (IC<sub>50</sub> 0.18 mg). The 5000 Da cut-off filtrates was performed systematic solvent extraction. D.W extracts of final step and butanol extracts showed high ACE inhibitory activity of 79.5% and 52.1%, respectively (Table 2). It suggest that the ACE inhibitor should be hydrophilic compound such as peptides. It is necessary further purification to characterize and elucidate structure-function relationship of the ACE inhibitor.

The HMG-CoA reductase inhibitory activity and tyrosinase inhibitory activity were 8% to 20% higher than those

**Table 1.** Physiological functionalities of the biomass from *Pichia anomala* KCCM 11473 grown on ginseng-steaming effluent (GSE) (Unit: %)

| Biomass                       | Angiotensin I-converting enzyme inhibitory activity | Fibrinolytic activity (u) | HMG-CoA reductase inhibitory activity | Glutathione S-transferase activity | Nitrite scavenging activity | Antioxidant activity | SOD-like activity | Tyrosinase inhibitory activity |
|-------------------------------|---|---------------------------|---------------------------------------|------------------------------------|-----------------------------|----------------------|-------------------|--------------------------------|
| Biomass <sup>a</sup> from GSE | 72.0 ± 1.0  | n.d <sup>b</sup>          | 46.5 ± 5.0                            | 100.0 ± 1.0                        | n.d                         | n.d                  | 13.0 ± 3.0        | 33.0 ± 1.0                     |
| Biomass from YEPD             | 51.2 ± 1.0  | n.d                       | 25.0 ± 5.0                            | 106.0 ± 1.0                        | n.d                         | n.d                  | n.d               | 25.0 ± 3.0                     |

<sup>a</sup>Biomass which were obtained from cultivation in GSE and YEPD medium, respectively.

<sup>b</sup>n.d : not detected.

**Table 2.** Angiotensin I-converting enzyme inhibitory activity of various extracts by ultrafiltration and systematic solvent extraction of the biomass from *Pichia anomala* KCCM 11473 (Unit: %)

| Ultrafiltration |              | Serial solvent extraction |                    |                      |                 |             |  |
|-----------------|--------------|---------------------------|--------------------|----------------------|-----------------|-------------|--|
| 5000 Da below   | 5000 Da over | Hexane extract            | Chloroform extract | Ethylacetate extract | Butanol extract | D.W extract |  |
| 61.6            | 5.8          | n.d <sup>a</sup>          | n.d                | 6.9                  | 52.1            | 79.5        |  |

<sup>a</sup>n.d : not detected.

**Table 3.** Enzyme activities of the biomass from *Pichia anomala* KCCM 11473 grown on ginseng-steaming effluents (Unit: U/ml)

| $\alpha$ -Amylase | Glucoamylase | Inulinase | Invertase | $\beta$ -Galactosidase | Protease        |                  |                    |
|-------------------|--------------|-----------|-----------|------------------------|-----------------|------------------|--------------------|
|                   |              |           |           |                        | Acidic (pH 3.0) | Neutral (pH 7.0) | Alkaline (pH 10.0) |
| n.d <sup>a</sup>  | n.d          | 0.6       | 0.9       | n.d                    | n.d             | 13.0             | 8.5                |

<sup>a</sup>n.d : not detected.

grown on the YEPD medium and YM medium (Kim *et al.*, 2002). However, the other functionalities were not determined or were lower than those of fibrinolytic activity (9.4%) and SOD-like activity (16.8%) of *Pichia anomala* and nitrite scavenging activity (47%) of the mutant, *P. anomala* HA-2 grown on YM medium (Kim *et al.*, 2002).

Overall, these results suggest that *Pichia anomala* grown on GSE may be beneficial in the production of a high-grade functional yeast biomass, which in turn could serve to eliminate pollution problems arising from GSE waste.

**Enzyme activity of the biomass.** Some industrial enzyme activities of the cell-free extract from the biomass were determined (Table 3). Neutral protease and alkaline protease activities were showed 13.0 U per ml and 8.5 U per ml, respectively. It suggest the GSE is valuable for application into enzyme industry such as detergent or medical industry. Meanwhile, invertase and inulinase activities were below 1 U per ml. The other enzyme activities were not detected.

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