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Cost-cutting of nitrogen source for economical production of cellulolytic enzymes by *Trichoderma inhamatum* KSJ1

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Abstract−For saccharifying food wastes, cellulolytic enzymes were produced using *Trichoderma inhamatum* KSJ1 in modified Mandel's medium. In a previous study, 0.1% bacto peptone in Mandel's medium was established as the best organic nitrogen source for the production of cellulolytic enzymes using strain KSJ1. However, economically, peptone was too expensive. Therefore, soybean, yeast and Chunggookjang (fermented soybean paste) were substituted for peptone in this research. Also, yeast or ground soybean hydrolyzed by sulfuric acid or from a culture broth of *Bacillus alcalophilus*, a strain producing protease, was added to the medium as the nitrogen source to the production of cellulolytic enzyme. In the cultivation using 0.5% yeast hydrolyzed with a culture solution of *B. alcalophilus* as the nitrogen source, the activities of FPase and amylase were 0.20 and 2.17 U/mL in a 100 mL flask, compared to 0.35 and 1.24 U/mL with the 0.1% peptone as control, respectively. In a 10 L jar fermenter, the activities of FPase and amylase were improved to 0.40 and 4.82 U/mL in the cultivation, respectively, using 0.5% yeast hydrolyzed with the culture broth, compared with 0.38 and 3.79 U/mL, respectively, for the 0.1% peptone as control. Therefore, hydrolyzed yeast was established as an available nitrogen source for the industrial scale production of cellulolytic enzymes by strain KSJ1, resulting in a 52.3% cost reduction in the production of cellulolytic enzyme by substitution of the expensive nitrogen sources.

Key words: Nitrogen Sources, Cellulolytic Enzymes, Cost-cutting, *Trichoderma inhamatum*

INTRODUCTION

Organic nitrogen sources, such as peptone, yeast extract and malt extract, are usually an expensive component of the microbial growth media in the production of enzymes. Within industry, minimizing the medium cost for the production of cellulolytic enzymes is a major problem.

Yeast, a byproduct from the beer industry, is generated in a huge amount annually in Korea. Moreover, soybean and Chunggookjang (fermented soybean paste) are well known for containing an abundance of protein components, which are not only low-priced nitrogen sources, but easily purchased also. Therefore, the aim of this study was to examine the potential of yeast, soybean and Chunggookjang as the nitrogen sources for the low-cost production of cellulolytic enzymes by *Trichoderma inhamatum* KSJ1.

In our laboratory, the cellulosic materials contained in food wastes were saccharified by using cellulolytic enzymes expressed as FPase (Filter Paperase), and the saccharified food wastes were used as carbon and energy sources of the fermentation for the production of BC (Bacterial Cellulose) [1] and ethanol [2,3]. BC and ethanol are known as functionally advanced material and a clean energy, respectively. The remaining solids generated from the saccharification process and the remaining solution after BC production culture was used for the fermentation of methane to reduce organic substances and recover the methane by anaerobic digestion. The slurry generated from the methane fermentation process was finally used as liquid fertilizer to complete the development of a total resource recovery system of food wastes that is zero-emission. Nevertheless, this system has not much advantage due to the high cost in the production of cellulolytic enzymes employed for saccharification of food wastes. In our laboratory, *Trichoderma inhamatum* KSJ1, which produces various cellulolytic enzymes, was isolated from rotten wood. The strain produced the enzymes in Mandel's medium substituted with cellulosic wastes, such as rice straw and paper wastes [1,4] as carbon and energy sources. However, until now, high-priced bacto peptone has been used as the nitrogen source. Thus, the expense of the nitrogen source accounts for a large portion of enzyme production. For the commercialization of enzymatic saccharification technology, the bacto peptone requires substitution with low-priced nitrogen sources. Accordingly, to greatly reduce the production cost of cellulolytic enzymes, the aim of this research was to substitute the high-priced bacto peptone with low-priced nitrogen sources, such as soybean, waste yeast from beer fermentation and Chunggookjang.

MATERIALS AND METHOD

1. Microorganisms

Trichoderma inhamatum KSJ1, a filamentous fungus, isolated from rotten wood and characterized by Kim [4], was used for the production of cellulolytic enzymes. *Bacillus alcalophilus*, a protease producing bacterium, used for hydrolyzing soybean and yeast, was supplied by the Korean Culture Center of Microorganisms (KCCM).

2. Substrates

Carbon sources: In our previous research, high-priced carbon sources in Mandel's medium were substituted with cellulosic wastes, such as rice straw and paper wastes [5]. The rice straw was cut into 2 cm using a chopper, soaked in tap water for one day, ground three

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Table 1. Elemental compositions of carbon and nitrogen sources

		N (%)	C (%)	H (%)	S (%)
Carbon source	Paper wastes	0.07±0.02	40.47±0.12	5.81±0.04	-
	Rice straw	0.29±0.07	40.26±0.17	5.39±0.09	-
Nitrogen source	Soybean	6.34	49.61	3.50	0.75
	Yeast	7.76	43.3	6.66	-
	Chunggookjang	6.92±0.09	52.79±0.07	7.78±0.01	-

times using a crusher, dried for 72 hours at 50 °C in drying oven, and finally homogenized for 2 minutes in a blender. Paper wastes were also produced by homogenizing 1.5×1.5 cm hand split sections of waste boxes for 3 minutes using a blender.

Nitrogen sources: soybean was used after homogenizing in a mortar, and then dried for 24 hours at 80 °C. Yeast from beer fermentation was used after drying for 24 hours at 80 °C, Chunggookjang was used in either the wet state or dry state (dried for 24 hours at 80 °C). The elementary compositions of the carbon and nitrogen sources are shown in Table 1.

3. Cellulolytic Enzyme Production

3-1. Cellulolytic Enzyme Production in Flask Level

T. inhamatum KSJ1 was initially pre-cultivated in a 500 mL baffle flask containing 100 mL of YMEB medium (yeast extract 4 g/L, malt extract 10 g/L, D-glucose 4 g/L), for 2 days at 30 °C on a shaking incubator at 120 rpm. 5% of the pre-cultured *T. inhamatum* KSJ1 was then inoculated into 100 mL of modified Mandel's medium for the production of cellulolytic enzyme, and cultivated at 30 °C and 120 rpm for 5 days in a 500 mL of baffle flask. Mandel's medium consisted of Avicel 5.0 g, CMC 5.0 g, bacto peptone 1.0 g, urea 0.3 g, $CaCl_2$ 0.3 g, KH_2PO_4 2.0 g, $(NH_4)_2SO_4$ 1.4 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg in distilled water 1.0 L. In the modified Mandel's medium, the Avicel and CMC were supplemented with 1% rice straw and 1% paper waste as the carbon sources. In the flask cultures, the observed cellulolytic enzyme activities were the average values of three replicates.

3-2. Cellulolytic Enzyme Production in 10 L Jar Fermenter

2% (100 mL) of the cultured *T. inhamatum* KSJ1 solution, pre-incubated at 30 °C and 120 rpm for 2 days in YMEB medium, was inoculated into 5 L of the Mandel's cellulolytic enzyme producing medium in a 10 L jar fermenter (BioG, Hanil R&D Co., Korea). The KSJ1 strain was incubated at 30 °C, with agitation and aeration rates of 200 rpm and 0.6 vvm, respectively, for 3.5 days. The Mandel's medium was supplemented with 1% rice straw and 1% paper wastes as the carbon sources and with substituted by soybean, waste yeast or Chunggookjang as the organic nitrogen source instead of peptone. During fermentation, pH was not controlled when peptone was used as the organic nitrogen source. However, with soybean or yeast hydrolyzed by protease solution cultivated by *Bacillus alcalophilus* as the nitrogen source, pH was increased above 6.0 during fermentation, because *B. alcalophilus* is an alkaline strain, so the pH of the culture broth was manually controlled between 5.0-5.5 by using 2 M of HCl and 2 M of NaOH.

4. Enzyme Activity Analysis

The activity of cellulolytic enzymes in the incubated in modified Mandel's medium was analyzed after centrifugation for 10 min at 12,000 rpm. The filter paper (FPase, Filter Paperase) activity, which describes the overall cellulolytic activity, was determined by the release of reducing sugars produced in 60 min from culture supernatant of 1.0 mL diluted with 50 mM citrate buffer of pH 4.8; 50 mg of Whatman No 1 disk filter papers was incubated in the reactant at 50 °C. Amylase activity in supernatant was analyzed by the release of reducing sugars produced in 30 min from the culture broth of 0.2 mL of an appropriately diluted enzyme, with 0.8 mL of soluble starch (1%) using the method of Thomas and Ji et al. [6]. One unit of enzyme activities was defined as the amount of enzyme that releases 1 μmol glucose per 1 minute, and reported as U/mL. The glucose equivalents (reducing sugars) generated during the assay were estimated by using 3,5-dinitrosalicylic acid (DNS method) [7], with glucose as the standard.

5. Substitution of Soybean, Yeast and Chunggookjang as Nitrogen Sources

For the substitution of the bacto peptone for low-cost nitrogen components, waste yeast from a beer factory, soybean or Chunggookjang were supplemented to the Mandel's medium instead of peptone. These nitrogen sources were added in the following four ways after the pre-treatment process: 1) homogenized yeast or soybean, 2) pre-treated yeast or soybean by pyrolysis or acid hydrolysis, 3) dried or wet Chunggookjang, and 4) yeast or soybean hydrolyzed using a protease solution cultivated by *B. alcalophilus*.

5-1. Substitution with Homogenized Yeast or Soybean

According to the precedent research results, the use of 1% rice straw and 1% paper wastes as the carbon sources, as well as 0.1% peptone as the organic nitrogen source was found to be the optimum for the production of cellulolytic enzymes by *T. inhamatum* KSJ1. For the substitution of the high-priced bacto peptone for soybean or yeast, the optimal substrate concentration was initially examined. Therefore, 0.1, 0.2, 0.5, 1.0 or 2.0% concentrations of soybean or yeast were added to the Mandel's medium instead of the 0.1% peptone. In flask cultures, *T. inhamatum* KSJ1 was pre-cultured for two days in 100 mL of YMEB medium at 30 °C and 120 rpm. 5% of the pre-cultured solution then was inoculated into the Mandel's medium and incubated for five days at 30 °C and 120 rpm.

5-2. Substitution with Yeast or Soybean Hydrolyzed by Sulfuric Acid

5 g of soybean or yeast was hydrolyzed using 3 M H_2SO_4 at 1.5 atm and 121 °C for 30 min, and pH then was adjusted to 6.0 using 10 M NaOH solution. The hydrolyzed soybean or yeast was added to the Mandel's medium as the nitrogen source at concentrations of 0.1, 0.2, 0.5, 1.0 and 2.0%.

5-3. Substitution with Chunggookjang

Chunggookjang is made from fermented soybeans using *Bacillus* sp., which is a nutritious and nitrogen-rich substance. The Chunggookjang, which was purchased from a specialty restaurant, had a

moisture content of 52.8% after drying at 80 °C for 24 hours. In flask cultures, Chunggookjang, in either the wet or dry state (80 °C, 24 h), was added to the Mandel's medium at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0% instead of bacto peptone as the organic nitrogen source. After incubating for five days at 30 °C and 120 rpm, the optimal substituent concentrations were determined by analyzing amylase and FPase activities. In a 10 L jar fermenter, 1% rice straw and 1% paper wastes were used as the carbon sources, and 0.2% dried Chunggookjang or 1.0% wet Chunggookjang was used as the nitrogen source. The amylase and FPase activities were measured after incubating for 3.5 days at 30 °C, 200 rpm and 0.6 vvm.

5-4. Substitution with Soybean or Yeast Hydrolyzed by Protease Solution

The protease-producing strain *B. alcalophilus* obtained from the KCCM was used. The incubation medium for the *B. alcalophilus* was an alkaline nutrient medium composed of beef extract 3.0 g, peptone 5.0 g, Na-sesquicarbonate solution 100 mL and agar 15.0 g in 1.0 L distilled water. After sterilizing the medium, 10% 1 M Na-sesquicarbonate solution was added to adjust the pH to 9.7. The composition of the Na-sesquicarbonate solution was: $NaHCO_3$ 4.2 g, Na_2CO_3 anhydrous 5.3 g and distilled water 100.0 mL. The strain was incubated for 1.5 days at 37 °C and 180 rpm in the alkaline nutrient medium by inoculating a stock solution mixed in a 1 : 1 ratio with glycerol at −70 °C. 25 g of soybean or yeast was then added to 200 mL of the *B. alcalophilus* solution and hydrolyzed at 37 °C and 180 rpm for 24 hours. The hydrolyzed soybean or yeast was uniformly mixed and then added to the Mandel's medium instead of peptone for the production of cellulolytic enzymes.

RESULTS AND DISCUSSIONS

1. Homogenized Yeast or Soybean as Nitrogen Source

When homogenized yeast or soybean was added to Mandel's medium as the nitrogen source, the amylase and FPase activities were measured to investigate possibility of substitution with soybean or yeast. In flask cultures using homogenized soybean or waste in the modified Mandel's medium, the amylase and FPase activities showed maximum values at concentrations of 0.5%. When 0.5% yeast was used as the nitrogen source, the amylase and FPase activities were 2.24 and 0.24 U/mL, respectively. When 0.5% soybean was added as the nitrogen source, the amylase and FPase activities were 1.55 and 0.25 U/mL, respectively. Conversely, when 0.1% peptone was used as the control, the amylase and FPase activities were 2.18 and 0.61 U/mL, respectively (Fig. 1). Therefore, it was concluded that waste yeast was a better nitrogen source than soybean. In a 10 L jar fermenter, when 0.5% waste yeast was supplemented to Mandel's medium in place of the peptone, the amylase and FPase activities were 0.25 and 0.01 U/mL, and with 0.5% soybean these values were 1.10 and 0.01 U/mL after 3.5 days of cultivation. As a result, homogenized soybean and waste yeast were found not to be suitable as the nitrogen source for the production of cellulolytic enzyme by *T. inhamatum* (Table 2).

Table 2. Comparison of amylase and FPase activities in 10 L jar fermenter when 0.5% of homogenized soybean or yeast was used as nitrogen source (cultivation conditions: 30 °C, 200 rpm, 0.6 vvm, 3.5 days)

	Amylase activity (U/mL)	FPase activity (U/mL)
Soybean (0.5%)	0.25	0.01
Yeast (0.5%)	1.10	0.01

2. Substitution with Soybean or Yeast Hydrolyzed by Sulfuric Acid as the Nitrogen Source

When homogenized soybean or yeast was added to the Mandel's medium, the activities of the cellulolytic enzymes were very low value. Therefore, they were hydrolyzed by using sulfuric acid and then added.

When the soybean or yeast was hydrolyzed with 3 M of sulfuric acid and was then added to the Mandel's medium as the organic and inorganic nitrogen sources, only small amounts of FPase and amylase were produced (Fig. 2). It was supposed that the Mailard reaction byproducts of soybean or yeast due to hydrolysis using sulfuric acid suppressed the growth of *T. inhamatum* KSJ1, which inhibited the production of cellulolytic enzymes.

3. Substitution with Chunggookjang as Nitrogen Source

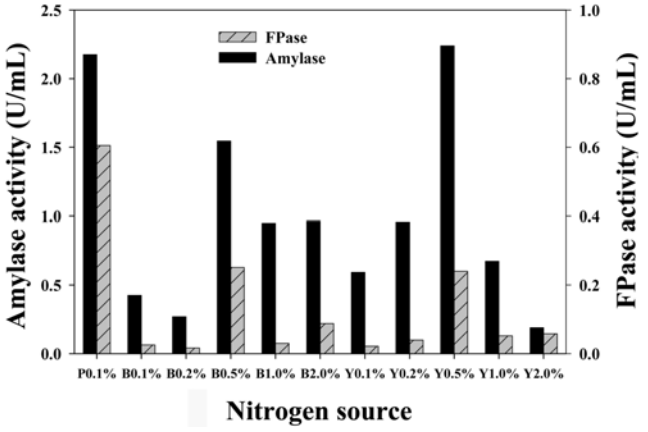

Fig. 1. Comparison of amylase and FPase activities in cultivation of substituting inorganic and organic nitrogen sources in 100 mL of Mandel's medium with homogenized soybean or yeast. P: Peptone, B: soybean, Y: Yeast (growth conditions: 30 °C, 120 rpm, 5 days).

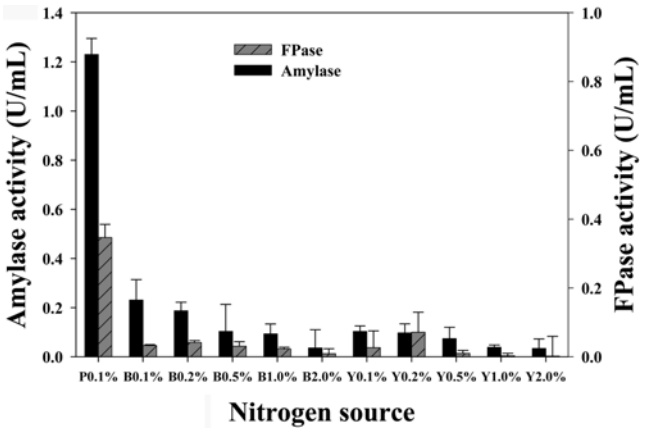

Fig. 2. Comparison of amylase and FPase activities in the cultivation using yeast and soybean hydrolyzed with 3 M H_2SO_4 in 100 mL of Mandel's medium. P: Peptone, B: soybean, Y: Yeast (growth conditions: 30 °C, 120 rpm, 5 days).

Table 3. Comparison of FPase and amylase activities with dry or wet state of Chunggukjang in Mandel's medium as nitrogen source in 10 L jar fermenter (growth conditions: 30 °C, 200 rpm, 0.6 vvm, 3.5 days)

	Amylase activity (U/mL)	FPase activity (U/mL)
Wet chunggookjang (1%)	1.01	0.02
Peptone (0.1%)	4.77	0.51
Dry chunggookjang (0.2%)	1.83	0.25
Peptone (0.1%)	3.12	0.44

The substitution of peptone by soybean or yeast, even when hydrolyzed by sulfuric acid, is almost impossible. Therefore, the use of nitrogen-rich Chunggookjang was examined.

In flask cultures, with the use of 0.2% dried Chunggookjang, the maximum amylase and FPase activities reached 0.70 and 0.02 U/mL, respectively, compared to 0.98 and 0.29 U/mL with 0.1% peptone (data not shown). In a 10 L jar fermenter, when 0.2% of dried Chunggookjang was used as nitrogen source, amylase and FPase activities were 1.83 and 0.25 U/mL, respectively, at 30 °C, 200 rpm and 0.6 vvm after 3.5 days of cultivation, which were only 65% of the control values. The amylase and FPase activities were 3.12 and 0.44 U/mL, respectively, when 0.1% peptone was used as the control (Table 3).

In flask cultures, with the use of 1.0% wet state Chunggookjang, the maximum amylase and FPase activities reached 1.27 and 0.11 U/mL, respectively, compared to 1.36 and 0.57 U/mL to the control values (Fig. 3). In a 10 L jar fermenter, when 1.0% wet Chunggookjang was added to the Mandel's medium, the amylase and FPase activities were 1.01 and 0.02 U/mL, but when peptone was used as the nitrogen source in the control, these activities were 4.77 and 0.51 U/mL, respectively (Table 3).

Therefore, it was considered that dry or wet state Chunggookjang in the place of peptone was not effective in the production of cellulolytic enzymes by *T. inhamatum* KSJ1.

3. Substitution with Soybean or Yeast Hydrolyzed by Protease Solution as Nitrogen Source

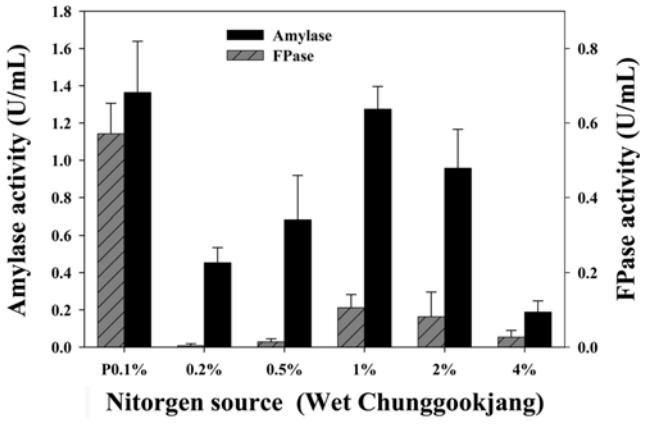

Fig. 3. Comparison of amylase and FPase activities in the cultivation using wet Chunggookjang in 100 mL of Mandel's medium instead of bacto peptone. P: Peptone (growth conditions: 30 °C, 120 rpm, 5 days).

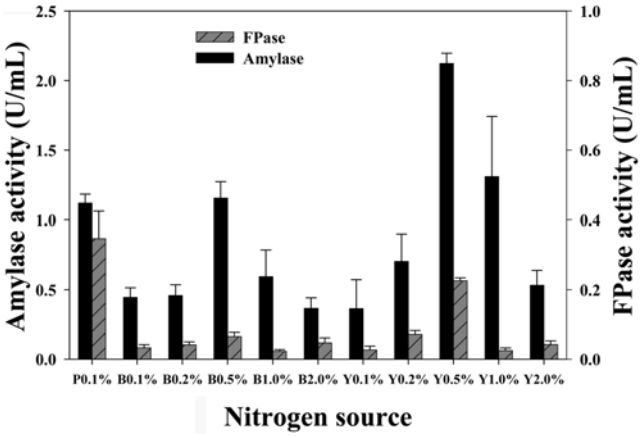

Fig. 4. Comparison of amylase and FPase activities according to different concentration of soybean and yeast hydrolyzed by culture broth of *B. alcalophilus* as nitrogen source in 100 mL of Mandel's medium. P: Peptone, B: soybean, Y: Yeast (growth conditions: 30 °C, 120 rpm, 5 days).

The substitution of peptone by soybean or yeast, even Chunggookjang is almost impossible. Therefore, soybean or yeast hydrolyzed by protease solution was examined.

The activities of the cellulolytic enzymes were higher in the hydrolyzed yeast than hydrolyzed soybean, with 0.5% concluded to be the optimal concentration. When hydrolyzed soybean was added, the amylase and FPase activities were 1.15 and 0.06, respectively, compared to 2.17 and 0.20 U/mL with hydrolyzed yeast in flask cultures. On the other hand, the amylase and FPase activities were 1.24 and 0.35 U/mL when peptone was used as the control (Fig. 4). Because the cellulolytic enzyme activities were higher with hydrolyzed yeast than in the control, it was considered as a reasonable substitute as the nitrogen source. Therefore, *T. inhamatum* KSJ1 was cultured on a larger scale in a 10 L jar fermenter at 30 °C, 200 rpm and 0.6 vvm for 3.5 days using Mandel's medium containing 0.5% yeast hydrolyzed by *B. alcalophilus* solution as the nitrogen source. During the cultivation, the pH was adjusted so as not to exceed 5.5 by the addition of 2 M of NaOH and 2 M HCl. The amylase

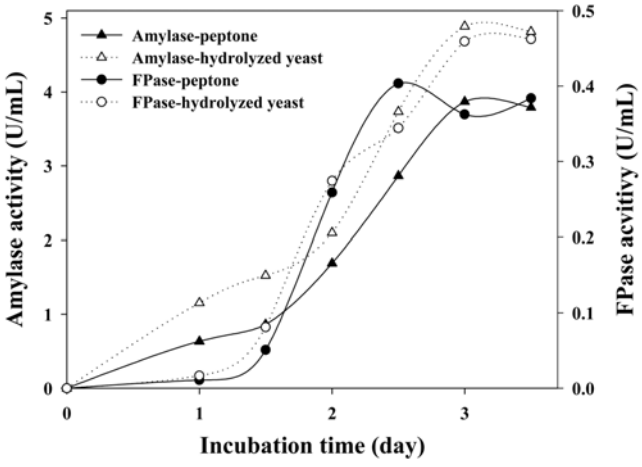

Fig. 5. Comparison of amylase and FPase activities with peptone or yeast hydrolyzed by *B. alcalophilus* culture broth in Mandel's medium as nitrogen source in 10 L jar fermenter (growth conditions: 30 °C, 200 rpm, 0.6 vvm).

Table 4. The unit cost of 1 L Mandel's medium for cellulolytic enzyme production

			Chemicals	Usage (g/L)	Unit cost (Won/kg)	Company	Unit cost (Won/L)			Percentage
Mandel's medium	Carbon source		Avicel	5	132,110	Fluka	660.6	736.6		77.8%
			CMC	5	15,200	Junsei	76.0			
	Nitrogen source	Inorganic	Urea	0.3	11,500	Junsei	3.5	189.1	23.1	20.0%
			Ammonium sulfate	1.4	14,000	DongYang Chemical	19.6			
		Organic	Bacto peptone	1	166,000	Difco	166.0		166.0	
	Others		Potassium phosphate monobasic	2	8,700	DongYang Chemical	17.4	21.3		2.2%
			Magnesium sulfate	0.3	5,100	Kanto Chemical	1.5			
			Calcium chloride	0.3	8,000	DongYang Chemical	2.4			
			Trace element	-	-	-	-			
	Total			-	-	-	-	946.9		100%
	Alkaline nutrient medium*		For 40 mL (in 1 L Mendel's medium)				56.0	56.0		

* In this study, alkaline nutrient medium was used for cultivation of protease producing strain *B. alcalophilus* for hydrolyzing yeast or soybean

and FPase activities were 4.82 and 0.46 U/mL, compared to 3.79 and 0.38 U/mL in the control (0.1% of peptone) (Fig. 5). Accordingly, not only at the flask level, but also on a 10 L scale, the possibility for the substitution of peptone for hydrolyzed yeast was verified.

4. Cost Reduction Effect in Cellulolytic Enzyme Production

In Mandel's medium, a cellulolytic enzyme production medium, CMC and Avicel were used as the carbon sources, with bacto peptone as the organic nitrogen source. By analyzing each composition of the Mandel's medium, the cost per tone was calculated to be 947,000 Won (Table 4). Of the cost, CMC and Avicel accounted for 77.8%, with peptone constituting 20.0%. According to the pre-researcher results, the cost of the Mandel's medium was reduced to 210,400 Won/ton when the high cost of carbon sources was substituted for the cellulosic wastes: rice straw and paper wastes. From this research, the cost of the medium for the production of cellulolytic enzyme was reduced by 52.3%, from 210,400 to 100,300 Won/ton, by substituting the bacto peptone for waste yeast (Table 4).

CONCLUSIONS

With the aim of recovering total resources from food wastes, this research focused on the inexpensive production of cellulolytic enzymes for saccharifying food wastes. The saccharified food wastes were used as the microbial cultivation medium for industrial fermentation. Therefore, for the mass production of low cost cellulolytic enzyme, bacto peptone should be substituted with inexpensive or waste nitrogen sources. In this study, yeast and soybean, homogenized or hydrolyzed by sulfuric acid or protease solution using *B. alcalophilus* and Chunggookjang, either in the wet or dry state, were substituted for bacto peptone as the nitrogen sources. As a result, on the addition of 0.5% yeast hydrolyzed using a protease solution as the nitrogen source in Mandel's medium, the cellulolytic enzyme activities showed maximum values in flask cultures. In a 10 L jar fermenter, the addition of 0.5% yeast hydrolyzed using protease solution, the amylase and FPase activities were 4.82 and 0.46 U/mL, respectively, compared with 3.79 and 0.38 U/mL for the peptone as control. Therefore, the addition of hydrolyzed yeast to the medium was established as an alternative to peptone as a nitrogen source in the production of cellulolytic enzymes. The substitution of waste yeast for peptone in Mandel's medium resulted in a 52.3% cost reduction in the production of cellulolytic enzymes.

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