Advance Journal of Food Science and Technology 5(5): 551-556, 2013
DOI:10.19026/ajfst.5.3125
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: November 19, 2012 Accepted: January 17, 2013 Published: May 05, 2013

Research Article

Utilization of *Candida utilis* Cells for the Production of Yeast Extract: Effects of Enzyme Types, Dosages and Treatment Time

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Abstract: The purpose of this study was to establish an enzymatic hydrolysis process to prepare yeast extract with the advantages of low-cost and high-content of flavor nucleotides. Yeast extract was produced from the broken cell suspension of *Candida utilis*, using papain, 5′-Phosphodiesterase (RP-1) and Adenosine Monophosphate (AMP) - deaminase. The effects of types, dosages and treatment time of enzymes on the recovery of solid, protein and flavor nucleotides, as well as the extract composition were investigated. Enzyme types remarkably affected the recovery of protein and solid and papain was found to be the most effective hydrolysis enzyme. The optimal dosage of papain and its treatment time were determined as 0.2% and 6 h, respectively. On this condition, the recovery of solid and protein of yeast cells was 69.26 and 60.87%, respectively. Further treatments with RP-1 (0.045%, 3 h) and AMP-deaminase (0.045%, 2 h) were employed to obtain a higher content of flavor 5′-nucleotides (GMP + IMP, 4.39%). This process had the advantages of a small amount of enzymes dosage, short enzymatic reaction time and high extraction yield.

Keywords: 5′-phosphodiesterase, AMP-deaminase, *Candida utilis*, papain, yeast extract

INTRODUCTION

Yeasts are beneficial in human health and are widely used in the production of alcoholic beverages and baking. Yeast cells, which are Generally Recognized as Safe (GRAS) and used as an inexpensive nitrogen source, contain plenty of protein, lipid, RNA, vitamins and minerals (Walker, 1998; In *et al*., 2005). Due to their nutritional characteristics, yeast cells are a source for the production of Yeast Extract (YE) which can enhance or impart a meaty flavor to food products. In food industry YE are widely used for the preparation of soups, gravies, meat products and sauces and in the flavoring of snacks (Dziezak, 1987; York and Ingram, 1996; Bekatorou *et al*., 2006).

YE can be manufactured by the breaking down of cells using endogenous or exogenous enzymes, otherwise stated as autolysis and hydrolysis. Autolysis is a degradation process carried out by activating the yeast's own endogenous enzymes to solubilize the cell components (In *et al*., 2005; Tanguler and Erten, 2008). This process has been used in many factories because of its relative low cost. However, autolysis has some disadvantages such as low extraction yield, difficulty in solid-liquid separation due to high content of residue in autolysate, poor taste characteristics as a favor enhancer and risk of deterioration due to microbial contamination. Enzymatic hydrolysis, on the other hand, is carried out as a safety process by cell wall lysis enzyme. Proteolytic enzymes or nucleic acid enzymes are widely used to break down insoluble macromolecules like proteins and nucleic acids into soluble products of peptides, amino acids, nucleotides and amino acid derivates (Nagodawithana, 1992; Stam *et al*., 1998). Previous studies showed that the controlling of cell wall degrading by β(1-3), β(1-6) glucanase, mannase and kitanase was rather difficult (Boonraeng *et al*., 2000), while sulfhydryl proteases derived from plants were quite effective, especially papain, which could assure a high hydrolysis efficiency (Conway *et al*., 2001; Boonraeng *et al*., 2000). In addition, using 5′-phosphodiesterase, Ribonucleic Acid (RNA) in the yeast cells can be degraded into four 5′-nucleotides: 5′-Guanosine Monophosphate (5′-GMP), 5′-Uracil Monophosphate (5′-UMP), 5′-Cytosine Monophosphate (5′-CMP) and 5′-Adenosine Monophosphate (5′-AMP). Moreover, using AMP-deaminase, 5′-AMP can be further convert into 5′-Inosine Monophosphate (5′-IMP) (Chae *et al*., 2001), since 5′-IMP and 5′-GMP are famous flavor enhancers (Jean *et al*., 2003).

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Candida utilis is thought as the most interesting microorganisms for its high contents of protein and nucleic acids (Lee and Kim, 2001; Rosma *et al*., 2005; Bekatorou *et al*., 2006). In this study, the food-grade *Candida utilis* was used for the production of YE. After cell disruption of *Candida utilis*, proteolytic enzymes were employed to hydrolyze intact proteins into small molecular peptides and amino acids, followed by the utilization of RP-1 to hydrolyze RNA for the release of 5′-nucleotides. The last step was to covert the 5′-AMP resulted from RNA degradation into 5′-IMP by AMP-deaminase. The whole process was optimized according to the effects of enzyme types, enzyme dosages and treatment time on solid and protein recovery.

MATERIALS AND METHODS

Microorganism: *Candida utilis* (CICC 1769) was purchased from China Center of Industrial Culture Collection (CICC) and was maintained on slants of the agar medium (glucose 2%, peptone 2%, yeast extract 1% and agar 1.5%; (w/v)) at 4°C.

Enzymes: Papain was purchased from Tianjin Noao Sci. and Tech. Development Co., Ltd., China. β-Glucanase was a gift from Sunson Industry Group Co., Ltd., China. YN-TL (Contains a neutral protease, produced by *Bacillus subtilis* fermentation, capable of lysing yeast cells by hydrolysis of the proteins in the yeast cell wall); RP-1 and Deamizyme 50000 were gifts from Amano Enzyme Group Co., Ltd., Japan. All enzymes were food-grade.

Preparation of yeast material: Yeast materials were prepared (Lee and Kim, 2001) in a 5 L fermenter (Shanghai Baoxing Bio-Engineering Equipment Co., Ltd., China) with a working volume of 3 L (glucose 3%, ammonium sulfate 0.5%, potassium dihydrogen phosphate 1%, magnesium sulfate heptahydrate 0.1% and calcium chloride dihydrate 0.05%) at 28°C for 20 h. The pH value was automatically controlled at 5.6 using 30% ammonia water. Foam was controlled using 10% antifoam. The aeration and agitation rates were maintained at 5 L/min of air and 400-600 rpm, respectively. Five percent of inoculum from the flask culture was used as seed culture. At the end of fermentation, the broth was centrifugated at 4°C for 5 min at 5000 g, then the supernatant was removed and the precipitation was washed twice using distilled water, the final cells were freeze-dried and stored at -20°C until use.

Enzymatic hydrolysis:

Pretreatment of yeast material: Dried yeast was suspended in 200 mL distilled water at a concentration of 15% (w/w) and incubated in a water bath at 95°C for 5 min. Then the inactivated yeast suspensions were treated under 900-1000 bar for twice using a homogenizer (APV2000, Rannie and Gaulin Homogenizers, Denmark).

Cell wall lysis enzymes and protease treatment:

The effect of enzyme types: β-Glucanase, YN-TL and papain were compared under their respective optimal conditions. (0.2% (w/v)) β-Glucanase was added in the broken cell suspension of yeast (10 mL) at an initial pH of 5.0 and was incubated at 50°C for 6 h. The hydrolysis using YN-TL was carried out under the same condition except that the pH was 7.0. The hydrolysis using papain was carried out under the same condition except that the pH was 7.0 and the incubation temperature was 55°C. The broken cell suspension of yeast treated in the same way with no added enzymes was used as control.

The effect of papain dosages: After the initial pH was adjusted to 7.0 by NaOH (5 M), the broken cell suspension of yeast (10 mL) was incubated at 55°C and papain was added into at a dosage of 0, 0.1, 0.2, 0.4 and 0.6%, respectively. Then the solutions were shaked at 150 rpm for 6 h.

The effect of treatment time: To determine the optimal treatment time of papain, papain was added into 10 mL broken cell suspension of yeast with the optimal dosage, then were shaked at 150 rpm and 55°C for 0, 2, 4, 6, 9 h, respectively.

The effects were evaluated based on the recovery of solid and protein. To determine the solid and protein contents of the hydrolysate, 2 mL of reaction mixture were heated at 95°C for 5 min to inactivate enzyme first and after a 20 min's centrifugation (10000 g, 4°C), the supernatant were freeze-dried and stored at -20°C until use.

Nuclease treatment: Nuclease treatment was carried out after the hydrolysis with papain (0.2%, 55°C for 6 h), the papain was inactivation and pH value was adjusted to 5.0. The dosage of RP-1 (5′-phosphodiesterase) was investigated at different concentrations of 0, 0.015, 0.03, 0.045%, 0.06 and 0.1% to hydrolyze RNA, respectively. The nuclease treatment was performed at 70°C for 3 h. To determine the optimal time of RP-1 treatment, 0.045% RP-1 was added into the hydrolysate and the mixture was kept at 70°C for 1, 2, 3, 4, 5, 6 h, respectively.

The effects of the dosages and treatment time of nucleases were evaluated according to the nucleotide content. To determine nucleotide content of the hydrolysate, pretreatment was implemented as follow: 2 mL of hydrolyaste sample was heated at 95°C for 5 min to inactivate enzyme and was centrifuged (10000 g, 4°C) for 20 min. At last, the supernatant was freeze-dried and stored at -20°C until use.

After treatment by papain and RP-1 under optimal conditions, the reaction mixture was sequentially treated by Deamizyme (0, 0.015, 0.03, 0.045, 0.06 and 0.1%, respectively) at pH 5.6 and 50°C for 2 h. The hydrolysates were then heated at 95°C for 5 min and centrifuged (10000 g, 4°C) for 20 min and the

supernatant was freeze-dried and stored at -20°C for further analysis.

Analytical methods: Total crude protein content (TN×6.25) of yeast materials and YE were estimated based on Total Nitrogen (TN) measurement by the Kjeldahl method. The contents of lipid and ash were determined by a method of previous report (Jie *et al*., 2012). Amino acid composition of YE was analyzed by ionexchange chromatography using Hitachi L-8900 automatic amino acid analyzer (Tokyo, Japan). Samples were hydrolysed by 6 M HCl (containing 5% thioglycolic acid) at 120°C for 45 min under the protection of nitrogen. The samples were hydrolysed by 4.2 M NaOH to determine the content of tryptophan; the samples were treated by formic acid to determine the content of cystine. Free amino acid composition was determined by Hitachi L-8900 Amino Acid Analyzer and the samples were not hydrolysed (Jie *et al*., 2012).

Total RNA of the strain was measured according to the method of previous report (Chuwattanakul *et al*., 2011) with minor modification. The yeast was resuspended with 5 mL of 0.5 M perchloric acid ($HClO_4$) and the suspension was incubated in ice-water for 30 min, then the suspension was centrifuged at 3000 g for 10 min. The obtained precipitate was resuspended in 5 mL of 0.5 M $HClO_4$, incubated at 70°C for 20 min and centrifuged at 3000 g. The supernate was collected for the measurement of RNA content. Ribonucleic acid (from torula yeast, Sigma, USA) was used as standard.

The contents of 5′-nucleotides (5′-GMP and 5′-IMP) were analyzed by Agilent 1200 series HPLC system (Agilent 1200 series, Agilent Technologies, USA). After filtered through a 0.22 μm membrane, the sample (20 μL) was injected into a SinoChrom ODS-BP column (5 μm, 4.6×150 mm, Dalian Elite Analytical Instruments Co., Ltd., China) and was eluted by phosphate buffer (0.02 M, pH 5.6) at 30°C with a flow rate of 0.5 mL/min for 30 min. The signals were detected at 254 nm.

Statistical analysis: All determinations were conducted in triplicate and all results were calculated as mean±Standard Deviation (SD). The differences were calculated by One-Way Analysis of Variance (ANOVA) using the statistical Software (SPSS, version 14.0, SPSS Inc., Chicago, USA). Differences were considered significant if $p<0.05$.

This study was conducted and finished on April 2012 in Tianjin Institute of Industrial Biotechnology, China Academy of Science.

RESULTS AND DISCUSSION

Effects of different cell wall lysis enzymes and protease treatment on the recovery of solid and protein: The effects of different cell wall lysis enzymes (β-glucanase, YN-TL and papain) on the recovery of solid and protein of yeasts were investigated at first.

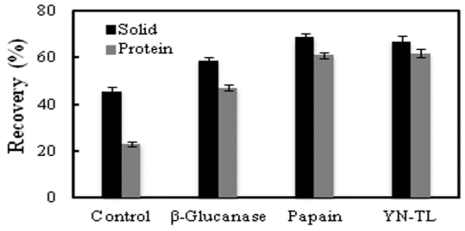

Fig. 1: Effects of different enzymes the on recovery of solid and protein
The dosages of enzymes were 0.2% and hydrolysis was performed for 6 h; All the dosages of enzymes were on a solid weight basis and each result represented the mean value from triplicate measurements

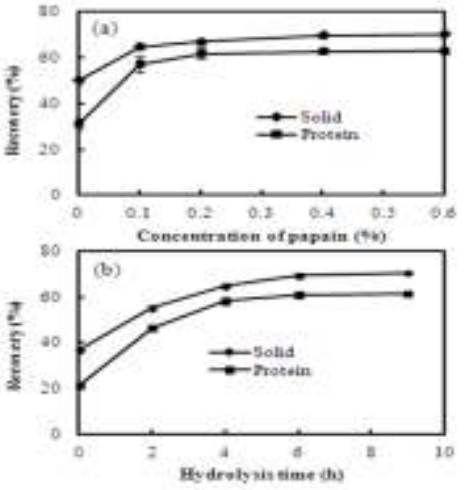

Fig. 2: (a) Effects of dosages of papain on the recovery of solid and protein. Hydrolysis was performed on 55°C, pH 7.0 for 6 h, (b) Effects of hydrolysis time on the recovery of solid and protein. The dosage of papain was 0.2% and hydrolysis was performed on 55°C, pH 7.0 for 0, 2, 4, 6, 9 h, respectively, all the dosages of enzymes were on a solid weight basis and each result represented the mean value from triplicate measurements

The results were showed in Fig. 1. The recovery of solid and protein of yeasts cells treated by β-glucanase was evidently lower than those treated by YN-TL or papain. The recovery of solid (68.6±2.01%) and protein of yeasts cells (61.07±1.44%) treated by papain were nearly equal to those treated by YN-TL (66.9±2.36% of solid recovery and 61.89±1.67% of protein recovery). In the study of Dolińska *et al*. (2012), papain was the most effective for the production of yeast hydrolysates. Similarily, papain showed the highest hydrolysis efficiency in our experiment. Besides, papain was much cheaper than YN-TL and β-glucanase. Therefore, it is a better choice for factories in YE production.

Based on the above discussions, the effect of the papain dosage and its reaction time on YE production was studied. As Fig. 2a revealed, the solid recovery and

Table 1: Chemical composition of yeast materials and YE

Items	Crude protein (%)	Total nucleic acid	Lipid (%)	Ash (%)	GMP+IMP (%)
Yeast material	31.1±0.10	16.80±0.36	9.7±0.10	6.4±0.10	ND
YE	32.7±0.19	15.14±0.61	0	9.1±0.10	4.39±0.04

ND: Not detected; Yeast material were prepared in a 5L fermenter, the yeast materials were freeze-dried and stored at -20°C until use; YE was prepared by yeast materials using combined enzymes

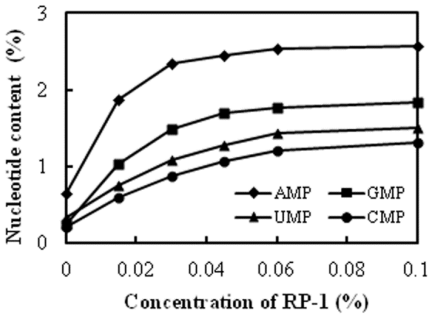

Fig. 3: Effects of concentration of RP-1 on the nucleotides content in hydrolysates
The hydrolysis was performed at 70°C, pH 5.0 for 3 h; All the dosages of enzymes were on a solid weight basis and each result represented the mean value from triplicate measurements

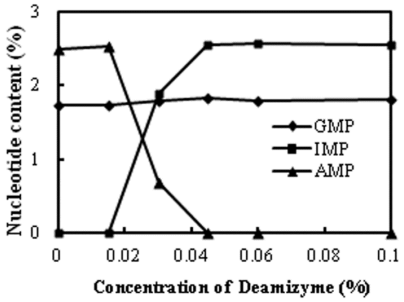

Fig. 4: Effects of dosage of deamizyme on IMP and AMP
The hydrolysis was performed at 50°C, pH 5.6 for 2 h; Each result represented the mean value from triplicate measurements

protein recovery of the control sample were very low (50.02 and 30.84%, respectively), however, with an addition of 0.1% papain, the recovery was significantly improved, indicating that papain degraded insoluble proteins and glycoproteins into soluble proteins, peptides and amino acids effectively. But the increase of papain dosage over 0.2% provided no more improvement to the solid and protein recovery. Therefore, the optimal dosage of papain was determined as 0.2% and the recovery of solid and protein under this condition was 67.02 and 61.62%, respectively. In this study, the dosage of papain was lower in comparison with some researches on the YE production. In the report by Vukasinović *et al*. (2007), the optimal dosage of papain of was 2.5% and the maxium solid recovery was 59.84%. The optimal dosage of protease reported by Chae *et al*. (2001) was 0.6% and under this dosage the highest of solid recovery was 53.1%.

The influence of incubation time on the recovery of solid and protein was shown in Fig. 2b. The soluble components accumulated with the prolongation of the reaction and the recovery of solid and protein peaked at 69.26 and 60.87% respectively after 6 h. In previous reports, the treatment time of protease for production of YE was in the range of 16-24 h (Boonraeng *et al*., 2000; Chae *et al*., 2001; Dolińska *et al*., 2012), which was much longer compared to ours; furthermore, the recovery of solid in those reports were 50-60%, which were also lower than ours. So that, the small dosage of papain and the short treatment time in our study possessed remarkably advantages in industrial application (Vukasinović *et al*., 2007).

Optimization of nuclease treatment: To determine optimum nuclease concentration for 5′-GMP formation, the hydrolysate prepared by 0.2% of papain for 6 h was subsequently treated with RP-1 at different concentrations (0.015-0.1% on a solid weight basis) for 3 h. The contents of 5′-AMP, 5′-GMP, 5′-UMP and 5′-CMP in the hydrolysate were determined by HPLC and the results were shown in Fig. 3. The overall nucleotides content rose with the increase of nuclease dosage and peaked at the enzyme RP-1 dosage of 0.045%. To be specific, the contents of 5′-UMP and 5′-CMP basically didn't rise anymore after the dosage of RP-1 exceeded 0.045%. Therefore, the optimal RP-1 dosage was found to be 0.045%. At that point the content of 5′-AMP and 5′-GMP were 2.45 and 1.70%, respectively.

In order to determine the optimal treatment time of RP-1, the hydrolysis using 0.045% RP-1 was performed at 70°C and pH 5.0 for 1, 2, 3, 4, 5 and 6 h, respectively. GMP content no longer increased after 3 h (not shown). Therefore, the optimal RP-1 treatment time was found to be 3 h.

AMP-deaminase (Deamizyme 50000) was used to convert 5′-AMP into 5′-IMP after the treatments of cell disrupting, papain (0.2% for 6 h) and RP-1 (0.045% for 3 h). The changes in the contents of 5′-GMP, 5′-AMP and 5′-IMP were analyzed. As shown in Fig. 4, during the treatment of Deamizyme, the 5′-GMP content maintained a constant as 1.79%, while 5′-IMP accumulated at the expenses of the consumption of 5′-AMP. When the dosage of Deamizyme reached 0.045%, the IMP content peaked at 2.64%.

Chemical composition of *C. utilis* CICC1769 and its YE: The compositions of yeast material and YE prepared by the combined treatment of papain, RP-1 and deamizyme, were summarized in Table 1. The

Table 2: Free amino acid and amino acid composition of YE

Amino acids	Amino acid (g/100 g YE)	Free amino acid (g/100 g YE)
Isoleucine	1.10	0.53
Leucine	1.57	0.83
Lysine	2.07	0.85
Cystine	0.30	0.25
Methionine	0.38	0.12
Phenylalanine	1.00	0.39
Tyrosine	1.48	0.62
Threonine	1.57	0.28
Tryptophan	0.20	0.10
Valine	1.36	0.50
Alanine	1.98	0.93
Arginine	4.05	2.35
Aspartic acid	1.99	0.05
Glutamic acid	3.75	0.68
Glycine	1.24	0.31
Histidine	0.47	0.22
Serine	1.38	0.25
Total	25.89	9.26

In the analysis of amino acid, YE samples were hydrolysed by 6 M HCI or 4.2 M NaOH before measurement; In the analysis of free amino acid, YE samples were analyzed directly

crude protein content (31.1%) of our yeast material was rather lower than the protein content (45-50%) of yeasts in previous reports (Boonraeng *et al*., 2000; Chae *et al*., 2001; Bekatorou *et al*., 2006; Dolińska *et al*., 2012); this poor protein content induced rather low protein content (32.7%) of YE. However, the relative high total nucleic acid (16.8%) of yeasts material resulted in the relative high flavor nucleotides (GMP+IMP, 4.39%), which was higher than the results obtained by Kim *et al*. (1999) and Chae *et al*. (2001), who reported the flavor nucleotide content as 3.2 and 3.67%, respectively. The lipid and ash contents of yeast material were determined as 9.7 and 6.4%, respectively. The ash content in YE was 9.1% and no lipid was detected.

In addition, the free amino acid and amino acid composition of YE were listed in Table 2. Free amino acids in the extracts were known to exert a major influence on the flavor. Some researchers pointed out that yeast hydrolysates obtained using papain yielded higher concentrations of valine, methionine, leucine, threonine and phenylalanine (Boonraeng *et al*., 2000; Vukasinović *et al*., 2007) and the sulfur amino acids would be better to enhance flavor, so the YE prepared by papain has better flavor. The total free amino acid of YE was 9.26 g/100 g and most essential amino acids were observed in the YE.

CONCLUSION

In this study, papain showed the highest hydrolysis efficiency for yeast cells. Its optimal dosage and treatment time were found to be 0.2% and 6 h, respectively. Under this condition, a relative high solid recovery (69.26%) and protein recovery (60.87%) were obtained. The optimal concentrations of RP-1 and deamizyme were both found to be 0.045% and their optimal hydrolysis time was found to be 3 and 2 h, respectively. The YE prepared under the optimal treatment conditions contained the highest content of free amino acids (9.26 g/100 g YE) and flavor nucleotides (GMP+IMP, 4.39%). In conclusion, the treatments combining cell wall broken, protease and nuclease hydrolysis in the production of YE, had the advantages of a small amount of enzyme dosage, short enzymatic reaction time, high extraction rate and a great deal of flavor nucleotides. This progress can be applied to industry for the production of YE from *Candida utilis*.

ACKNOWLEDGMENT

The study was supported by the National High Technology and Research Development Program (2012AA021403), Major scientific and technological program of the Tianjin Binhai New Area (2011-BK120045) and the Academy-Locality Cooperation Project of Chinese Academy of Sciences (DJBJ-2011-019).

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