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Production of Hydrolysates by *Actinomucor elegans* During Soy Protein Deep-liquid Fermentation

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ABSTRACT

Production of hydrolysates by *Actinomucur elegans* during soy-protein deep-liquid fermentation was a promising prospects for future applications in the protein hydrolysate industry. And the optimal incubation conditions for protease production by *Actinomucor elegan*, which have been investigated in several studies. The hydrolyzing conditions were optimized by the single factor and orthogonal tests. Five single factor of important effect on the DH and PCL were explored. The optimum condition was determined as fermentation time 5 days, substrate concentration 3%, initial pH 6.0, liquid medium volume 80 (mL bottle^{-1}), Inoculums concentration 4.5 (v/v). The maximum DH can reach 38.29%.

Key words: Hydrolysates, fermentation, soy protein

INTRODUCTION

Protein hydrolyzation is a common food process utilized for the production of flavor enhancers and bitter peptides are frequently generated during enzymatic production of functional, bioactive protein hydrolysates or during the aging process of fermented products such as sufu (Adler-Nissen, 1986). Extensive hydrolysis of protein is required for hydrolyzed vegetable protein production to obtain a high amino acid level. Carefully designed partial hydrolysis may be acceptable for hospital diets. Appearance, solubility, flavor and biochemical safety of the product vary with the degree of hydrolysis. Currently, formation of chlorohydrins like 3-chloro-1, 2-propanediol (MCPD) and 1,3-dichloro-2-propanol (DCP) in HVP production due to strong acid hydrolysis has been a serious concern in many countries to use the HVP clinically (Collier *et al*., 1991). Partial hydrolysis of some proteins, especially soybean protein, produces a strong bitter taste.

The formation of bitter peptides during partial hydrolysis of casein and soybean protein and its control have been the subject of extensive studies (Adler-Nisen, 1984; Clegg and McMillan, 1974). The mixture of endo-peptidase and exo-peptidases has beem used for food protein hydrolysis in order to reduce the bitterness of the hydrolysate as well as to increase the degree of hydrolysis (Izawa *et al*., 1997; Parker and Pawlett, 1986). Defatted soybean flour was treated by mild-acid and enzymatic hydrolysis (Lee *et al*., 2001) and wheat gluten was enzymatic hydrolysis by the combination of Alcalase™, Flavourzyme™ and peptidase NP-2™ which giving high yields of soluble protein (Hong, 1997).

Carboxypeptidases from *A. elegans* preferred hydrophobic synthetic substrates, such as Z-Phe-Leu, Z-Phe-Tyr-Leu and Z-Phe-Tyr and carboxypeptidases from *A. elegan* were efficient tools for decreasing the bitterness of peptides due to liberate the fewest free amino acids, which consisted of 73% hydrophobic amino acids, under acidic conditions (Fu *et al*., 2011).

Actinomucor elegans has been commonly used as a starter in the manufacture of sufu, a traditional Chinese fermented soybean curd that resembled a soft creamy-type cheese and has been produced in China for more than 1,000 years (Han *et al*., 2001). During sufu fermentation, *A. elegans* proteases (Han *et al*., 2003) catalyze the degradation of proteins into low molecular weight peptides and amino acids (Chou and Hwan, 1994), which contribute some special flavors and textures to the products (Wang *et al*., 1974). Enzymatic de-amidation offers several advantages over chemical methods, providing reaction selectivity and milder de-amidating conditions by using a neutral pH and room temperature (Hamada and Marshall, 1988).

In this study, the hydrolyzation of Soy Protein Concentrate Flour (SPCF) was investigated by using *Actinomucor elegans* fermentation treatments in order to efficiently produce a non-bitter protein hydrolysate for proteins. The optimum condition of *Actinomucor elegans* fermentation was determined.

MATERIALS AND METHODS

Organism and culture conditions: *Actinomucor elegans* (AS 3.2778) was provided by College of Food Engineering and Biotechnology, Tianjin University of Science and Technology (Tianjin, China). Stock culture of *A. elegans* was stored on Potato Dextrose Agar (PDA) slants at -20°C. Seed culture medium and fermentation medium composition were 30 g SPCF, 5 g D-glucose, 2 g $MgSO_4$, 2 g KH_2PO_4, 1 g yeast extract and distilled water up to 1000 mL. Each 250 mL conical flask was filled 80 mL medium solution mentioned above. The pH of the solutions was kept nature and then autoclaving (121°C, 20 min). The spore suspension was prepared by adding 10 mL of sterile distilled water to a slant culture that had just been taken out of the incubator (28°C) on the third day of cultivation. One conical flask added 4 mL spore suspension (1 mL containing about 5×10^6 viable propagules mL^{-1}) before putting on the Numerical show Vibration Cultivating Box (28°C, 1 day). Then, add different percentage (v/v) of the seed medium to the fermentation medium and put them on the Numerical show Vibration Cultivating Box (28°C).

Materials and chemicals: Soy Protein Concentrate Flour (SPCF) containing 65.0% protein wad used to make different concentration (%, w/w) protein dispersion in distilled water. SPCF is a gift from Jining Nature Foods Co., Ltd (Shang dong, China).

Determination of degree of hydrolysis: After fermentation procedure, the enzyme reaction of the hydrolysates were terminated by heating at 105°C for 15 min and then centrifuged at 4000 rpm for 20 min. The α-amino nitrogen content was measured by Formol titration method using 0.1 N NaOH solution (AACC, 1983). DH is defined as the percentage of cleaved peptide bonds:

$$DH = \frac{h}{h_{tot}} \times 100\%$$

where, h_{tot} is the total number of peptide bonds per protein equivalent and h is the number of hydrolyzed bonds. α and h_{tot} are 0.342 and 0.78, respectively (Adler-Nissen, 1986). The DH was

calculated by the amount of nitrogen in the supernatant among the total nitrogen determined by Kjeldahl method. The average peptide chain length was estimated from DH (Adler-Nissen, 1986). Briefly, degree of hydrolysis was computed as equation as follows and the average Peptide Chain Length (PCL) was calculated:

$$PCL = \frac{1}{DH}$$

$$DH(\%) = (\frac{A}{B} - a)/htot \times 100\%$$

where, A is the α-amino nitrogen content and B is the total nitrogen in the sample (mg mL^{-1}).

Statistical analysis: Statistical calculations were carried out by SPSS V.18.0 software. p-values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Effect of fermentation time on protein hydrolysis: Different fermentation time will affect a reaction. Figure 1 shows effect results of different fermentation time on DH and PCL. Because, it needs some time to thallus growing for *Actinomucor elegans*, in the start production the content protease is very little and protease activity is not high. That is the main reason for hydrolysis degree little. With the time prolonged, the biomass grown rapidly along with the activity of protease; After 5 days, *Actinomucor elegans* protease activity basic no longer change and the ability of enzyme production began to drop and the DH reached maximum value (36.81%). Therefore, the fermentation time was determined at 5 days.

Effect of initial pH on protein hydrolysis: The pH value was often different in the stage of fungus growth and metabolism synthesis. It was a key factor to determine the growth condition of fungus that the fermented liquid was acidity or alkaline. And the shifty pH value will affect the

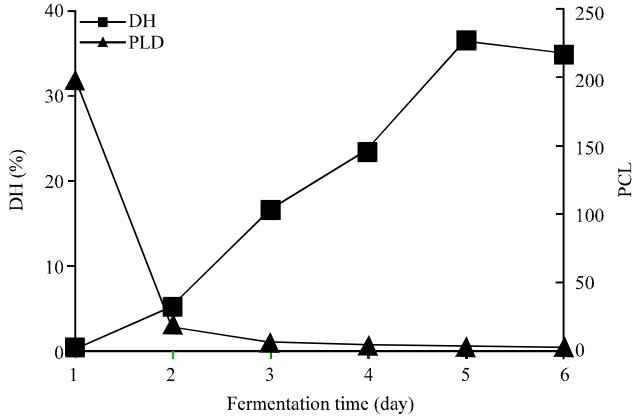

Fig. 1: Degree of hydrolysis of a substrate concentration of 3% SPCF, Fermentation of Continuous cultivation at a temperature of 28°C using *Actinomucor elegans* for six days and measured the DH every other day

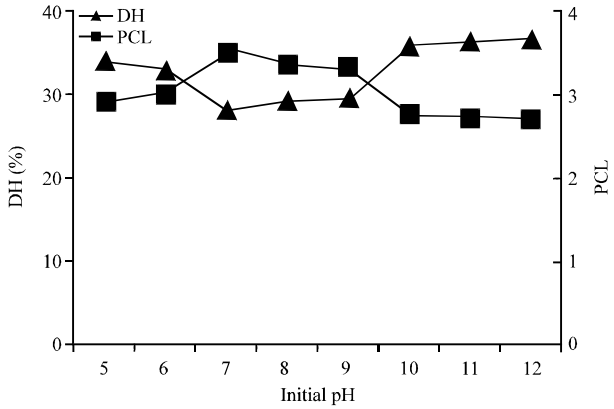

Fig. 2: Effect of initial pH on protein hydrolysis, Substrate concentration of 3% SPCF and fermentation of continuous cultivation at a temperature of 28°C using *Actinomucor elegans* for five days and then measured the DH, Square stands for DH and triangle stands for PCL

growth of fungus so that choose appropriate pH is important (Chi *et al*., 2012). In the process of fermentation soybean protein using *Actinomucor elegans*, it used a mixture of several enzyme including acid protease, neutral protease and alkaline protease (Zheng and Zhao, 2008). In the initial culture medium pH value of 7.0, the DH of hydrolysates reached the highest value. In the pH 6.0 culture condition, it was very suitable for growing in the liquid for *Actinomucor elegans* (Wang *et al*., 2003). In Alkaline medium (pH 9-12), the composite enzyme showed lower DH, may due to the condition was not appropriate for *A. elegans* and so it is in Acidic condition (pH 5-6). The change of hydrolysis degree as shown in Fig. 2, the DH was the largest in pH value 7. This may due to that the strain activity was closely related to the pH value of environment. The pH value of environment influence the growth of strain and thus influence the protein transform rate and the fermentation process.

Effect of substrate concentration on protein hydrolysis: Effect results of different substrate concentration on DH and PCL were shown in Fig. 3. When the stains were inoculated into the fermented liquid, the substrate concentration was a determined factor to the normal growth for *Actinomucor elegans*. The substrate concentration of SPCF was high leading to a big viscosity and the high growth rate and oxygen consumption. The contradiction of supply oxygen will influence later bacteria normal growth and metabolic pathways. We can see DH of the liquid supernatant was decreased along with the increase of concentration of fermented liquid. And the DH was biggest when the substrate concentration was 1%. This was different form Li *et al*. (2002) who reported that the best concentration of soy protein isolate was 3%. The reason was that the different substrate specificity and inoculums concentration and fermentation period.

Effect of inoculums concentration (v/v) on protein hydrolysis: In order to try to shorten the fermentation time and achieve the required hydrolysis degree, it is necessary to inoculation amount spores liquid of *Actinomucor elegans*. The fungus grows slow and has weak resistance to the environment when the inoculums concentration was lesser. And the premature aging fungus was disadvantageous to the fermentation processing. On the other hand, when the inoculums concentration was more, the fungus grows too fast which making the culture medium viscosity

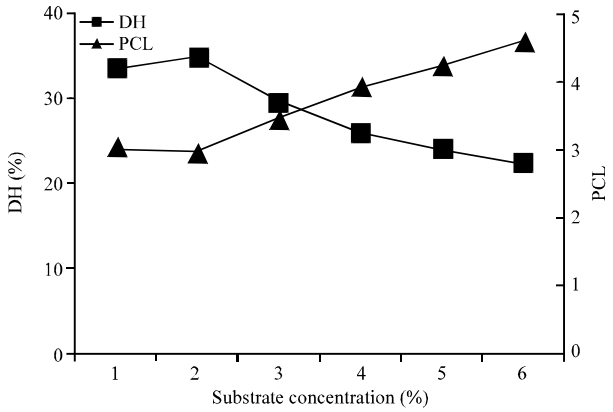

Fig. 3: Effect of substrate concentration on protein hydrolysis. Substrate concentration of 3% SPCF and continuous fermentation cultivation at a temperature of 28°C using *Actinomucor elegans* for five days and then measured the DH

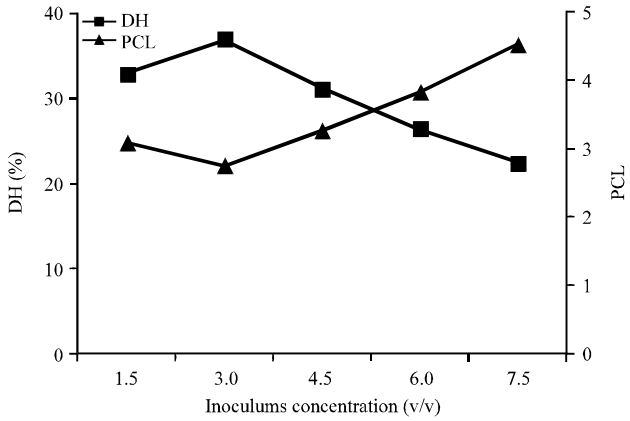

Fig. 4: Effect of inoculums concentration (v/v) on protein hydrolysis. Substrate concentration of 3% SPCF and continuous fermentation cultivation at a temperature of 28°C using *Actinomucor elegans* for five days and then measured the DH

increases and oxygen also is restricted. And it could also lead to a part of fungus to autolysis to influence the fermentation processing. As can be seen from Fig. 4, the DH increased quickly as the inoculums concentration (v/v) changed from 1.5 to 3.0 and decreased after 3.0. The possible reason is that inoculums concentration (v/v) was used as reactant beneficial to hydrolysis reaction. However, over-inoculums concentration (v/v) led to too difficult touch of substrate and inoculums concentration (v/v), which could be unbeneficial to a fermented reaction. Therefore, the result we confirmed inoculums concentration (v/v) at about 3% as appropriate. The result was different from (Li *et al*., 2002), who reported the best inoculums concentration was 4%. The reason was that Li *et al*. (2002) used seed fermentation liquid while in this research we used spores liquid.

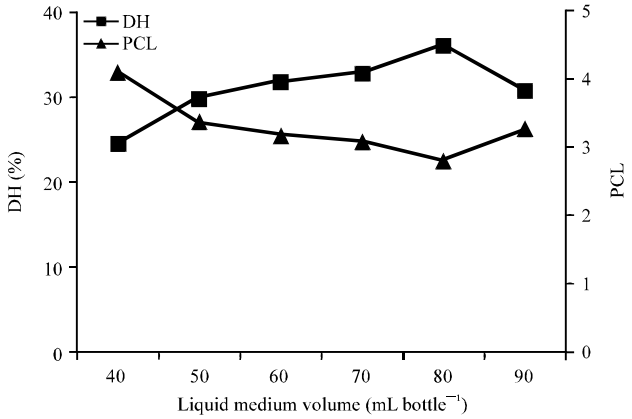

Fig. 5: Effect of liquid medium volume on protein hydrolysis, Substrate concentration of 3% SPCF and continuous fermentation cultivation at a temperature of 28°C using *Actinomucor elegans* for five days and then measured the DH

Table 1: Coded and real values for each variable of the orthogonal experimental design

		Coded levels		
Variables	Symbol	1	2	3
Substrate conc. (%)	A	2	3	4
Initial pH	B	6	7	8
Liquid medium volume (mL bottle^{-1})	C	70	80	90
Inoculums conc. (v/v)	D	1.5	3.0	4.5

Effect of liquid medium volume on protein hydrolysis: The liquid medium volume influences the strain growth period and oxygen in the bottle. In this paper, we explored the different liquid medium volume of 40, 50, 60, 70, 80, 90 mL per 250 mL triangle bottle. The best result was 80 mL per 250 mL triangle bottle as shown in Fig. 5.

Optimization of hydrolysis parameters: In this experiment, we took DH values produced in the process of fermentation as objective parameters. The fermentation time was fixed as 5 days. The influence of substrate concentration (A), Initial pH (B), liquid medium volume (C) and inoculums concentration (D) on the hydrolysis by *A. elegans* was determined using One-factor experimental design as mentioned in the previous section. Each variable was coded at three levels: 1, 2 and 3 (Table 1).

The observed values for DH at different combinations of the independent variables are presented in Table 2. The significance of the investigated factors and levels could be realized using the Range (R) analysis method. As can be seen, in the light of DH values of fermentation, the best reaction condition was $A_2B_1C_1D_3$. As can be seen from the ANOVA table (Table 3), the significance of factors for DH followed the sequence: A>C>B>D. Among the independent variables substrate concentration and liquid medium volume had relatively significant effect ($p \leq 0.05$). We confirmed the optimal fermentation condition was fermentation time 5 days, substrate concentration 3%, initial pH 6.0, liquid medium volume 80 (mL bottle^{-1}), Inoculums concentration 4.5 (v/v). In the end, the maximum DH can reach 38.29%.

Table 2: Orthogonal design for experimental design and actual results

	Code values				Real values				
Run	A	B	C	D	A	B	C	D	DH (%)
1	1	1	1	1	2	6	70	1.5	34.74
2	1	2	2	2	2	7	80	3.0	36.15
3	1	3	3	3	2	8	90	4.5	34.42
4	2	1	2	3	3	6	80	4.5	38.29
5	2	2	3	1	3	7	90	1.5	34.71
6	2	3	1	2	3	8	70	3.0	31.93
7	3	1	3	2	4	6	90	3.0	32.72
8	3	2	1	3	4	7	70	4.5	30.38
9	3	3	2	1	4	8	80	1.5	33.00

Table 3: Analysis of variance (ANOVA) for the orthogonal experiment

Source	SS	D_f	MS	F-value
A	18.104	2	9.052	19.446*
B	7.208	2	3.604	7.742
C	18.027	2	9.013	19.363*
D	0.931	2	1.136	1.000
Error	0.93	2		

*$p<0.05$, $F(2, 2) = 19.00$

CONCLUSION

This study provided the optimal condition for fermentation soybean protein using *Actinomucor elegans*. Amino nitrogen and DH (hydrolysis degree) was determined as screen indictor. The preliminary study confirmed that fermentation time, substrate concentration, initial pH, liquid medium volume (mL bottle^{-1}) and inoculums concentration (v/v) was the main factor for DH. Through the orthogonal experimental design, we confirmed the optimal fermentation condition was fermentation time 5 days, substrate concentration 3%, initial pH≥6.0, liquid medium volume 80 (mL bottle^{-1}), Inoculums concentration 4.5 (v/v). The maximum DH can reach 38.29% and therefore, *A. elegans* displays promising prospects for future applications of in the protein hydrolysate industry.

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