International Biodeterioration & Biodegradation 104 (2015) 112–117

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Optimization of hydrolysis condition of blood meal by *Bacillus subtilis* with response surface methodology

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ARTICLE INFO

Article history:
Received 23 September 2014
Received in revised form
21 May 2015
Accepted 21 May 2015
Available online 11 June 2015

Keywords:
Bacillus subtilis
Blood meal
Degree of hydrolysis
Degradation
Response surface methodology

ABSTRACT

The effects of initial pH, inoculum size, rotation speed, degradation temperature and time on the degree of hydrolysis (DH) by *Bacillus subtilis* CICC 10265 which were generated by ultra-violet (UV) in blood meal degradation medium were evaluated in single-factor experiments. The degradation conditions were optimized through response surface methodology by implementing the Box-Behnken design based on single-factor experiments. The optimal conditions for hydrolysis were a degradation temperature of 33.8 °C, a degradation time of 69.36 h, a pH value of 7.0, an inoculum size of 7.2×10^7 CFU/mL and a rotation of speed 250 r/min. Under these conditions, the model predicted a DH of 62.18%, and it was experimentally determined to be 62.05% under the optimal conditions for degradation. Three main influencing factors are degradation temperature, degradation time and initial pH.

1. Introduction

Blood is a major byproduct of abattoirs in the meat industry and is produced in large volumes (Zhang et al., 2014) but is commonly not fully utilized or even discarded (Wang et al., 1997). The open sustems in many industrial abattoirs other than closed collection systems are applied in many industrial abattoirs and the blood is frequently contaminated by contact with microorganisms and airborne bacteria (Toldrà et al., 2004), which brought biosafety issues. So for preventing spoilage organisms or even pathogens from growing, some hygienic precautions must be taken for safety during collection. However, according to some studies (Jamroz et al., 2011; Chen et al., 2013; Almeidaa et al., 2013), these biosafety issues can be avoided as much as possible. Thus the blood from animals that pass both ante and post mortem inspections can be processed as products for animal and human (Dàvila Ribot, 2006). Blood meal has a crude protein content of at least 80% and a low production cost, it has considerable potential to be used in animal feed (Goedeken et al., 1990; Hansen et al., 1993; Kats et al., 1994; Bergstrom et al., 1997). However, blood meal is not extensively used in feed due to its poor palatability, low digestion rate, amino acid imbalance and other shortcomings. Compared with other blood meal processing methods, microbial degradation effectively improves the shortcomings of blood meal for use as a source of protein (Chen et al., 2003; Fu et al., 2003; Li, 2003; Yang et al., 2006). The degradation industry has experienced considerable development in China. The digestion and absorption of proteins, cellulose, pectin and organic acids by animals are effectively improved by microbial degradation. Complex molecules, such as macromolecular proteins, were degraded into small peptides and other small-molecular-weight substances. A large quantity of bacterial proteins was produced by the degradation of blood meal, counterpoising the amino acid contents of the blood meal. After degradation, blood meal was sufficient for the nutritional requirements of animals. Degradation technology is conducive to the widespread use of blood meal in animal husbandry (Zheng et al., 2014). For high-level production of neutral protease we developed a strain, via UV mutagenesis, using *Bacillus subtilis* CICC 10265 as parent strain in this study. After being processed, the digestibility of the protein, which replete with FAAs and peptides of various molecular weights, is improved (Guo et al., 2007; Gaskell and Smith, 2007), and the product is easily absorbed (Rust, 1995).

2. Materials and methods

2.1. Microorganism and blood meal

A mutant strain of *B. subtilis* strain 10265, which was obtained by ultraviolet radiation exposure, was obtained from the China

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http://dx.doi.org/10.1016/j.ibiod.2015.05.018
0964-8305/

Center of Industrial Culture Collection. The blood meal was obtained from the WuJi County blood meal factory (Heilongjiang Province, China).

2.2. Strain activation and preparation of the seed culture medium

The bacteria were cultivated in slant medium at 37 °C for 12 h and then inoculated in the seed medium at 37 °C for 12 h.

2.3. Media and cultivation

The medium used for blood meal degradation was composed of the following: 50 g/L swine blood meal (smashed through a 60-mesh sieve) and 50 g/L wheat bran (smashed through a 60-mesh sieve). The pH of the medium was adjusted according to the experimental design. The microorganism was cultured in 250 mL Erlenmeyer flasks containing 50 mL of the medium. The Erlenmeyer flasks were incubated on a rotary shaker.

2.4. Liquid-state degradation

The culture medium was incubated with inoculum size of 1.8×10^7 CFU/mL (seed culture). After mixing well, the inoculated medium was incubated at 37 °C for 48 h with rotating speed of 150 r/min. The flask was incubated in boiling water for 15 min to inactivate the enzyme. Then, the liquid medium in the flask was removed by filtration using filter paper. The filtrate was diluted to 100 mL with distilled water to determine the DH.

2.5. Single-factor experiments

The DH was calculated from the free-NH2. Each single-factor experiment was conducted five times to determine, (1) inoculum size (1.8×10^7 CFU/mL, 3.6×10^7 CFU/mL, 5.4×10^7 CFU/mL, 7.2×10^7 CFU/mL, 9.0×10^7 CFU/mL, 1.08×10^8 CFU/mL, 1.26×10^8 CFU/mL) by using initial pH, 7.0; rotation speed, 150 r/min; degradation, 37 °C for 48 h followed by 50 °C for 6 h (2) rotation speed (0 r/min, 50 r/min, 100 r/min, 150 r/min, 200 r/min, 250 r/min, 300 r/min) by using inoculum size, 1.8×10^7 CFU/mL, initial pH, 7.0; degradation temperature, 37 °C; degradation, 37 °C for 48 h followed by 50 °C for 6 h (3) the initial pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0)by using the hydrolysis conditions were the following: inoculum size, 1.8×10^7 CFU/mL; rotation speed, 150 r/min; degradation, 37 °C for 48 h followed by 50 °C for 6 h (4) the degradation temperature (25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C) by using the hydrolysis conditions were the following: initial pH, 7.0; inoculum size, 1.8×10^7 CFU/mL; rotation speed, 150 r/min; degradation at different temperatures for 48 h followed by 50 °C for 6 h (5) the degradation time (12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h) by using the hydrolysis conditions were the following: initial pH, 7.0; inoculum size, 1.8×10^7 CFU/mL; rotation speed, 150 r/min; degradation, 37 °C for different incubation times followed by 50 °C for 6 h.

2.6. Determination of optimum degradation conditions

The SPSS Statistics software (Version 19, 2010; IBM, USA) was used to compare the F values between groups based on the single-factor experiments, resulting in the identification of three main factors, as shown in Table 1. Based on a Box-Behnken central composite design (BBD), response surface methodology was used to optimize the degradation of swine blood meal (Tanyildizi et al., 2005; Lucio et al., 2011; Xiu et al., 2011).

2.7. Determination of the degree of protein hydrolysis

The ninhydrin colorimetric method was used to determine the DH (Lowry et al., 1951; Pericin et al., 2009). The standard curve for free-NH_2 was determined in the laboratory as follows: $A = 0.011A-0.007$, $R^2 = 0.979$.

2.8. Statistical analysis

The data from the Box-Behnken design, which are shown in Tables 2 and 3, were used to determine the regression coefficients of the second-order multiple regression models. The Design-Expert package (Version 7.0.0, 2005; Stat-Ease, USA) was employed for the regression analysis of the data and to estimate the coefficients of the regression equation. The statistical significance of the model was determined by applying Fisher's F-test. A canonical analysis was also performed to predict the shape of the curve generated by the model. The two-dimensional graphical representation of the system behavior, i.e., the response surface, was used to describe the individual and cumulative effects of the variables and the mutual interactions between the independent and dependent variables.

3. Results and discussion

3.1. The impact of inoculum size on the degree of protein hydrolysis

The results showed that there is almost no change in the DH with a gradual increase in the inoculum size from 1.8×10^7 CFU/mL to 1.26×10^8 CFU/mL. The inoculum size had no effect on the DH of the degradation broth. The increase in the production of protease using small inoculum sizes was suggested to be due to the higher surface area to volume ratio, which resulted in the increased production of protease (Rahman et al., 2005). Some researchers (Haq et al., 1993; Iqbal et al., 2010; Bansal et al., 2012) noted that lower enzyme biosynthesis at lower inoculum size is probably due to insufficient conidial cells to use the fermentation medium, while the decreased yield at higher inoculum size is probably due to anaerobic conditions and nutritional imbalance due to tremendous growth in the medium.

3.2. Impact of the rotation speed on the degree of protein hydrolysis

The results are shown in Fig. 1A. *B. subtilis* is an aerobic bacterium, and the rotation speed directly affects the amount of dissolved oxygen in the degradation broth. As shown in Fig. 1A, the degree of protein hydrolysis increases with an increase in the rotation speed. At a rotation speed higher than 250 r/min, the degree of protein hydrolysis did not exhibit an apparent increase. A rotation speed of 0 r/min corresponds to a static culture. The surface of the medium features a layer of plaque folds that does not adequately contact the degradation medium, resulting in a very low DH.

The higher agitation rates could increase the oxygen pressure of the system but did not bring about the increase in production,

Table 1
Analysis of variance of single-factor experiments.

Factor	Sum of squares	Df	Mean square	F value
Initial pH	492.214	6	82.0357	72.9644
Inoculum size	22.1396	6	3.68994	0.20233
Rotation speed	4048.56	6	674.759	92.6018
Fermentation temperature	8325.04	6	1387.51	301.893
Fermentation time	3730.09	6	621.682	260.997