

Particle size for improvement of peptide production in mixed-culture solid-state fermentation of soybean meal and the corresponding kinetics

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Abstract: Experiments were undertaken to study particle size and its distribution for improvement of peptide production by mixed-culture (*Bacillus subtilis*, *Saccharomyces sp.* and *Lactococcus lactis*) solid-state fermentation (SSF) of soybean meal and the corresponding kinetics. A particle size of 1-1.4mm of soybean meal gave the highest final peptide yield, and as the proportion of 1-1.4mm/1-2mm in natural soybean meal up to 61.30%/58.58%, the distribution of particle size was also appropriate for peptide production. Logistic model fitted the data most accurately and could be used for growth kinetic profiles during the course of fermentation, and the higher growth rate was calculated for the substrate with optimal particle size distribution. Hence the mechanism could be inferred that appropriate particle size did improve the according rate of microbial growth, thus leading to the higher peptide yield in soybean meal SSF within a limited fermentation time.

Keywords: Particle Size, Peptide, Soybean Meal, Kinetics, Solid-State Fermentation

1. Introduction

The need for animal feed will inevitably increase in the future. As a main protein source for animal feedstuffs, soybean meal often has several antinutritional factors including carbohydrates [1], and antigenic proteins [2], which might cause hypersensitivity. Microbial fermentation [3, 4] is a more economical approach to reduce the antinutritional factors of soybean meal, and the use of different strains for soybean meal fermentation, not only can produce microbial enzymes to reduce anti-nutritional factors in soybean meal, but also can accumulate some other beneficial metabolites such as peptides that exert specific positive effects on cultured animal cells [5]. Therefore, improvement of peptide production from soybean meal fermentation will provide a higher nutritional value for animal growth and also be interested by more and more researchers now.

Solid-state fermentation (SSF) is often used to process soybean meal for the lower cost of production. In SSF process, the moist solid substrate is used and without free-flowing water, and thus the adherence and penetration

of microorganisms are clearly associated with the physical properties of the substrate such as the crystalline or amorphous nature, the accessible area, surface area, porosity, particle size, etc. In all the above factors, particle size should play a major role due to the dependence of other factors on it [6]. Hence it is of interest to know how particle size affects the production based on the substrate availability during SSF. The effect of particle size on the growth and product formation in SSF was studied by many [7-9]. But until now almost no report has been available regarding the effects of the particle size and its distribution on the changes in the peptide production during mixed-culture SSF of soybean meal. Therefore an attempt was made in the present study, with particular reference to the effects of the particle size and its distribution on peptide production and growth profiles of soybean meal SSF.

2. Materials and Methods

2.1. Microorganisms and Culture Preparation

Bacillus subtilis (ACCC 01746), *Saccharomyces sp.*

(CICIM Y0362) and *Lactococcus lactis* (ACCC 11092) were provided by Henan Engineering Laboratory for Collection and Selective Breeding of Industrial Microorganisms (Zhengzhou, China). The strains were cultured on nutrient agar (peptone beef extract agar) plates for *Bacillus subtilis*, potato dextrose agar plates for *Saccharomyces sp.*, and MRS agar plates for *Lactococcus lactis*. The plates were incubated at 30 °C for 24 h for *Bacillus subtilis* and *Saccharomyces sp.* And 7 days for *Lactococcus lactis* until colonies produced, and were kept at 4 °C as cultures until use.

2.2. Solid Substrate and Chemicals

Soybean meal was produced by Henan Sunshine Oils and Fats Group (Zhengzhou, China), analysed for protein (46%, N×6.25) by Kjeldhal method, subjected to a sieving procedure using sieves mesh-size of 10, 14, 18, 40 and 80, and classified into six different diameter sizes: <0.18mm(P1), 0.18-0.425mm(P2), 0.425-1mm(P3), 1-1.4mm(P4), 1.4-2mm(P5), and >2mm(P6), according to which of the sieves mesh-size that the soybean meal retained. The soybean meal without treatment of sieving procedure was called natural soybean meal and expressed by “natural” in this study. The soybean meal with different particle sizes and its natural were used alone or in combination as solid substrates. All chemicals and medium ingredients were purchased from Shanghai Chemical Co. (Shanghai, China).

2.3. Solid-State Fermentation (SSF)

Two culture loops of *Bacillus subtilis* (B), *Saccharomyces sp.* (S) and *Lactococcus lactis* (S) were respectively transferred to 250 mL conical flasks containing 50 mL of liquid soybean meal dextrose medium made of 4% soybean-meal powder (particle size <0.15mm, passing through 100-mesh screen) and 1% dextrose, in order to adapt the strains for the solid substrate. The flasks were shaken at 150 rpm for *Bacillus subtilis* and statically placed for *Lactococcus lactis* and *Saccharomyces sp.* At 30 °C for 8 h. The solution was used as a stock solution for SSF.

A 250-mL conical flask, which was capped with rubber stopper perforated with a syringe needle for gas release, was used for SSF. A total inoculum of 10% (v/w) was used to inoculate 50 g of substrate with the inoculation ratio of B:S:L = 1:1:1 (v:v:v). Solid substrate with an original moisture content of 12% was adjusted to a moisture content of 50% by using distilled water, and the initial pH was maintained at a natural level as reported [15] and actually tested at 6.8±0.1 in this study. No nutrients or minerals were added to the substrate, due to soybean meal as the natural medium that contains the organic carbon, nitrogen, phosphorus, vitamins and metal ions to allow microbial growth, and the case that a supplement such as wheat bran might relatively reduce the total content of protein in soybean meal owing to the lower content of protein in supplement and thus would affect the nutrient value for

application in animal feed. In addition, for this scheme the effect of particle size of soybean meal on the SSF could be investigated clearly without the interference of the supplement. The substrate was sterilized at 121 °C and 103 kPa in an autoclave for 20 min. And, SSF was done by statically incubating the substrate at 30 °C in a thermostat incubator within a water reservoir to maintain the relative humidity and moisture level of the substrate, and the samples were withdrawn every 3 h. Content of peptide of the fermented soybean meal was measured. All treatments were repeated three times.

2.4. Peptide and Dry Matter Assays

The peptide content in sample was determined by Lowry method [16]. 1 g sample was put into 50 mL of 15% (w/w) TCA solution and stirred and incubated for 5 min at 25 °C, and then the mixture was filtered. After the filtrate was centrifuged at 4,000×g for 30 min, the obtained supernatant, containing peptide (< 10 kDa) from fermented soybean meal, could be analyzed by Lowry method. The content of small peptide, expressed as a percentage (dwb), was calculated by dividing the weight of TCA-soluble peptide by the dry matter mass in the original sample.

The dry matter mass was determined by weighing after oven-drying at 105 °C overnight.

2.5. Calculation

The linear, exponential and logistic equations have typically been used to characterize growth profiles in SSF [17]. The integrated forms of these equations are expressed as follows:

linear,

$$X = Kt + X_0 \quad (1)$$

exponential,

$$X = X_0 e^{\mu t} \quad (2)$$

and logistic,

$$X = \frac{X_m}{1 + (\frac{X_m}{X_0} - 1)e^{-\mu t}} \quad (3)$$

in which, X , biomass concentration; X_0 , X at time 0; t , fermentation time; K , growth rate; μ , constant of growth rate; X_m , carrying capacity. For the measurement of biomass (X) in the construction of growth profiles, some different units have been used by researchers [17], and in this study the content of peptide (dwb), produced by microorganisms in SSF, was used for the growth profile, due to the purpose of this study, relation of peptide production and particle size distribution, which could also reflect the microbial growth indirectly.

For the logistic equation, the maximum growth rate (dX/dt) can be obtained through the second derivative of Eq. (3) at,

$$\frac{d^2X}{dt^2} = 0$$

and the solution to this equation is,

$$t_{X_m/2} = \frac{\ln(\frac{X_m}{X_0} - 1)}{\mu} \quad (4)$$

at which, $X = X_m/2$. The point $(t_{X_m/2}, X_m/2)$ is the inflexion of the Eq. (3) for a sigmoidal curve, where the maximum rate occurred.

Eqs. (1) - (3) were fitted to triplicate sets of data using linear and non-linear regression modules of the software, Polymath (Version 6.0, USA). The correlation coefficients R^2 were used to judge whether the equation represents correctly the data and for comparing various equations representing the same dependent variable. The goodness of fit was evaluated with the root-mean-square deviation (Rmsd).

2.6. Statistical Analysis

Analysis of variance (ANOVA) and least significant difference (LSD) were used in order to determine the differences among treatments ($P < 0.05$) by SAS (version 9.1).

3. Results and Discussion

3.1. Effect of Particle Size of Substrate on the Peptide Yield of Soybean Meal SSF

During SSF process, the availability of surface area plays an important role in microbial attachment, mass transfer, subsequent microbial growth and metabolite production, and is most associated with the particle size of the substrate [10]. To study the effect of the particle size of substrate on

the peptide yield in SSF of soybean meal for 36h, different particle sizes ($<0.18\text{mm}$, $0.18\text{-}0.425\text{mm}$, $0.425\text{-}1\text{mm}$, $1\text{-}1.4\text{mm}$, $1.4\text{-}2\text{mm}$, $>2\text{mm}$, and natural) were used in the cultivation and the results are shown in Figure 1. The maximum of peptide content ($P < 0.05$) was obtained on the soybean meal with particle size of 1-1.4mm. An increase or decrease in the particle size led to reduction of peptide yield, but no significant difference ($P > 0.05$) was found between particle sizes of 1-1.4mm and 1.4-2mm, and between particle sizes of 0.425-1mm and $>2\text{mm}$. Fermentation of the fine ($<0.425\text{mm}$) soybean meal resulted in significantly lower content ($P < 0.05$) of peptide. Moreover, it was interesting that SSF with natural substrate had a high yield of peptide, only lower than treatments with particle sizes of 1-1.4mm and 1.4-2mm (Figure 1), which should be related to its high content (42%) of particle size of 1-2mm (Table 1) and would be concerned with particle size distribution of mixture.

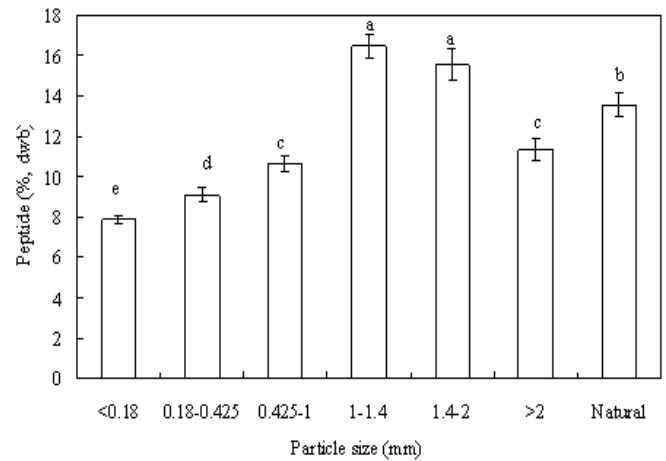


Figure 1. Effect of particle sizes on peptide contents in mixed-culture SSF of soybean meal for a fermentation time of 36h. $N = 3$. Means followed by the same letter are not significantly different at $P = 5\%$. $LSD_{0.05} = 0.9152$.

Table 1. Particle size distribution of different substrates.

Substrates	Particle size distribution (%)						
	P1	P2	P3	P4	P5	P6	1-2mm
Natural	3.00	17.00	30.00	22.00	20.00	8.00	42.00
Natural + P4 (w : w)							
M1(1:0.25)	2.25	12.75	22.50	37.60	15.00	6.00	52.60
M2(1:0.5)	2.00	11.30	20.00	48.00	13.30	5.30	61.30
M3(1:1)	1.50	8.50	15.00	61.00	10.00	4.00	71.00
M4(1:2)	1.00	5.70	10.00	74.00	6.70	2.70	80.70
Natural + P4 + P5 (w : w : w)							
M5(1:0.1:0.1)	2.50	14.17	25.00	26.66	25.00	6.67	51.66
M6(1:0.2:0.2)	2.14	12.14	21.43	30.00	28.58	5.71	58.58
M7(1:0.3:0.3)	1.87	10.63	18.75	32.50	31.25	5.00	63.75
M8(1:0.5:0.5)	1.50	8.50	15.00	36.00	35.00	4.00	71.00

3.2. Effect of Particle Size Distribution of Mixture on the Peptide Content during SSF of Soybean Meal

Natural mixed with other substrates with some particle size was investigated during the course of SSF and the selection of supplement was based on the results of previous batches. Substrate of P4 (1-1.4mm) was hence chosen and P5 (1.4-2mm) was also considered for comparison. The particle size distributions of different mixtures are shown in Table 1, and effects of mixtures on changes of peptide content with fermentation time can be seen from Figure 2. The increase of proportion of P4 or P4+P5 in the mixture could enhance the final yield of peptide. For the total fermentation process, the yield of

peptide increased significantly ($P < 0.05$) as the proportion of P4 and P4+P5 was above 61.30% and 58.58% respectively, compared to natural. However, for the similar type treatments such as M1, M2, M3 and M4, when the proportion of P4 and P+P5 was above 71.00% and 63.75% respectively, there was no significantly difference in peptide production between those mixtures (M3 and M4, M7 and M8) used as substrates. Moreover, no significantly difference between P4, M4 and M8, which indicated that the proportion of P4 and P4+P5 in mixture reaching 80.70% and 71.00% respectively had a similar peptide yield to substrate only with 1-1.4mm particle size.

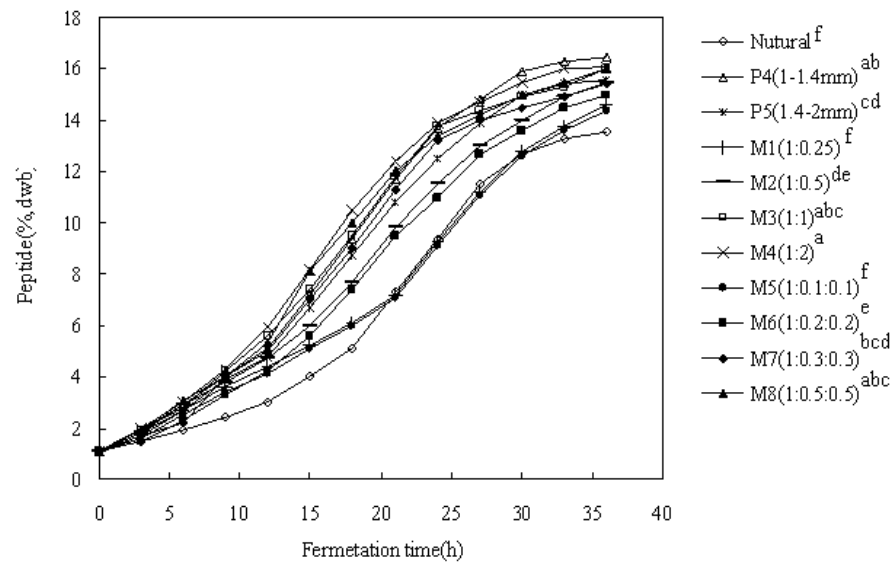


Figure 2. Time profiles of peptide content at different substrates during mixed-culture SSF of soybean meal. Different letters represent significant differences at $P = 5\%$. $LSD_{0.05} = 0.562$. All different substrates are described in Table 1.

3.3. Growth Kinetic Profiles

Table 2. Kinetic analysis of growth profiles using liner, exponential and logistic equation*.

S	Linear			Exponential			Logistic				
	K (% \cdot h $^{-1}$)	R^2	Rmsd	μ (h $^{-1}$)	R^2	Rmsd	X_m (% dwb)	μ (h $^{-1}$)	$t_{X_m/2}$ (h)	R^2	Rmsd
N	0.33	0.91	0.38	0.076	0.87	0.46	18.15ab	0.11e	24.9a	0.985	0.160
P4	0.47	0.97	0.28	0.083	0.62	0.96	17.59abc	0.16bc	16.9bcd	0.998	0.067
P5	0.43	0.97	0.25	0.081	0.66	0.86	16.82abc	0.15cd	17.7bc	0.998	0.057
M1	0.36	0.96	0.24	0.077	0.86	0.47	17.93a	0.12e	22.7a	0.992	0.110
M2	0.41	0.98	0.21	0.080	0.73	0.71	16.88abc	0.14d	19.0b	0.998	0.062
M3	0.45	0.97	0.27	0.082	0.56	0.98	16.46bc	0.17ab	15.5cd	0.998	0.070
M4	0.47	0.97	0.28	0.083	0.50	1.07	16.60abc	0.18a	14.7d	0.999	0.035
M5	0.35	0.96	0.27	0.077	0.87	0.45	17.96a	0.12e	22.4a	0.995	0.090
M6	0.39	0.97	0.25	0.079	0.77	0.66	16.79abc	0.14d	19.0b	0.999	0.049
M7	0.43	0.96	0.28	0.081	0.61	0.92	16.19c	0.16bc	16.4cd	0.997	0.081
M8	0.46	0.97	0.26	0.082	0.54	1.00	16.34c	0.17ab	15.5d	0.998	0.061

* S, Substrates; N, Natural; All different substrates are described in Table 1. Values followed by the same lowercase letter within a column are not significantly different at $P = 5\%$.

Growth kinetic profiles of different substrates including natural, substrate with single particle size, and mixture, were analyzed by fitting the data in Figure 2 to three empirical growth model (linear, exponential and logistic) and results shown in Table 2. The higher R^2 values were found in linear (0.91-0.97) and logistic (0.985-0.998) equations, indicating that these two models could represent correctly the data but logistic equation seemed to represent given sets of data much more correctly by further comparison. Therefore, exponential model would not be considered because the lower R^2 . Furthermore, the smallest Rmsd values (0.035-0.160) were found in logistic equation, also showing that this model fitted the data most accurately. So, logistic model would provide good answer for questions under investigation for mixed-culture SSF of soybean meal. This model fitting data from SSF with appropriate distribution of particle size (containing higher proportion of 1-1.4mm or 1-2mm) of mixtures (M3, M4, M7 and M8) or 1.4mm particle size of substrate had a smaller X_m value and a higher μ value. On the other hand, as the proportion of P4 and P4+P5 was above 61.30% and 58.58% respectively, the growth rate constant (μ) increased significantly ($P < 0.05$).

3.4. Discussion

It is known that the size of particle is directly associated with the specific surface area and porosity of the solid substrate. And in general, bigger particles, with less surface area, cause a trend towards poorer accessibility to the microorganism, but inter-particle porosity is greater, while smaller particles, with larger surface area, are preferred for better accessibility to the microorganism, but the porosity is less. These two opposing factors probably interact to determine the products corresponding to optimum microbial growth [11]. Furthermore, smaller particles are favorable for anaerobic fermentation, due to forming a higher solid medium density and a less void fraction. But, too small particles may diminish the heat transfer and carbon dioxide exchange rates because of medium compaction [12]. On the other hand, larger particles provide better aeration/respiration opportunities [13], which does encourage the growth of the aerobic organism. Thus, an appropriate particle size is required for a particular process in SSF [14]. In this study, three microorganisms were combination of aerobe (*Bacillus subtilis*) and facultative anaerobe (*Saccharomyces sp.* And *Lactococcus lactis*), and final results showed that appropriate particle size could make three microorganisms grow as quickly as possible, but otherwise microbial growth would be inhibited to some extent, thus leading to reduce the peptide yield. So, an optimal particle size of 1-1.4mm should be chosen for soybean meal SSF, which represented a compromise between the accessibility of nutrients and the availability of oxygen. In addition, particle size of 1.4-2mm might be considered, mainly owing to no statistically significant difference with particle

size of 1-1.4mm.

Results also suggested that the yield of peptide could be improved by adjusting the proportion of P4 (1-1.4mm) or P4+P5 (1-2mm) of natural substrate. But it was found that in the all process of SSF, P4 (1-1.4mm) treatment had overall higher peptide yield ($P < 0.05$) than P5 (1.4-2mm), other than previous batches, partly due to the different data obtained at fermentation time of 36h and 0-36h, respectively, thus leading to the smaller value of $LSD_{0.05}$ for comparison analysis of the latter, and this result also showed that the 1-1.4mm was the optimal particle size of the substrate in practice for all process of soybean meal SSF. In addition, numerically, SSF with M4 seemed to produce higher yield of peptide than that with P4, although statistically, no significant difference between both substrates. And the reason for these results might need further study to investigate the relation between growth profiles and particle size distributions.

A smaller X_m value and a higher μ value indicated a lower carrying capacity or biocapacity of substrate, which was the maximal load of substrate for peptide production and microbial growth that should be related to living environment, namely the particle size distribution in this study, and a higher growth rate, under those distributions. However, statistically, there was no significant difference between most X_m values obtained, partly due to a limited effect of particle size distribution on X_m and maybe other factors needing further study. In addition, the increase of the growth rate constant (μ) implied that the particle size distribution affected growth rate significantly, which was also showed from the data of the time to maximal growth rate ($t_{Xm/2}$): less time for higher proportion of P4 and P4+P5 (Table 2). Therefore, the mechanism could be inferred that appropriate particle size could improve the corresponding rate of microbial growth.

4. Conclusions

In all, the optimal particle size of soybean meal for peptide production by SSF was 1-1.4mm, and when the proportion of 1-1.4mm/1-2mm in natural soybean meal was increased to 61.30%/58.58% or more, the distribution of particle size was also appropriate for peptide production. Logistic model fitted the data most accurately, and showed a higher growth rate for the substrate with optimal particle size distribution in SSF. Hence the mechanism could be inferred that appropriate particle size could improve the corresponding rate of microbial growth, thus causing an increase of peptide yield in soybean meal SSF within a limited fermentation time.

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