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Selected Essential Amino Acid Enhancement by *Bacillus cereus* from Solid State Fermentation of Soy Pulp Through Carbon Concentration and Fermentation Period

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Abstract

Essential amino acids are important in aquatic animal growth rate. Hence, this study was carried out to find out the enhancement of five selected essential amino acid namely tryptophan, lysine, methionine, valine and isoleucine by carbon concentration and fermentation period in soy pulp for aquaculture uses. In the present study, fermentation medium containing (soy pulp, 95.5%; yeast extract, 2%; ammonium sulphate, 2%; 0.5% glucose v/v) were subjected to solid state fermentation for fermentation period screening (0 day, 4 days, 8 days, and 12 days) inoculated with *Bacillus cereus* (MH027625). To acknowledge, the soy pulp, yeast extract and ammonium sulphate is in powder form. Out of four fermentation periods, 8 days of fermentation was the best period of fermentation for maximum total essential amino acid enhancement (42.9 ± 19.5 gL^{-1}). This medium was further supplemented with different type of carbon sources (glucose, sucrose, and molasses) at 0.5% v/v to increase total enhancement of essential amino acid. The best carbon source (sucrose) was then further supplemented with different concentration (0%, 2%, 4%, 6%, 8%, and 10% v/v) to increase total enhancement of essential amino acid. Finally, total essential amino acids were successfully enhanced with 43.1 ± 1.63 gL^{-1} of production after 8 days of fermentation supplemented with 2% sucrose.

Keywords: Essential amino acid; Carbon source; Sucrose concentration; Fermentation period; Solid state fermentation

Introduction

Essential amino acid is one of main component in fish feed nutrition. In fish feed industry, the main source of essential amino acid is from fish meal [1]. However, the rise of fish meal cost become issue in fish feed industry due to the rise of fish cost. Therefore, the alternative source of essential amino acid should be discovered to solve the issue. Among economical protein source that can be considered is from plant. For instance, many studies have been done on the usage of soy pulp in fish feed industry [2]. Soy pulp is a by-product from soy milk production [3]. The soy pulp can be good source of essential amino acid due to high protein content. It was reported that soy pulp contains about 23% of protein [4], makes it potential source of essential amino acid in fish feed formulation. However, high anti-nutritional factor in plant protein make it barrier to use the soy pulp directly in fish feed formulation [5]. There are several ways decrease the level of anti-nutritional factor such as heat processing, roasting, extrusion, and micronization [6] including fermentation [7]. Fermentation has been done throughout many generations to obtain animal feed. Recent studies has been made to produce essential amino acid from another agro-industrial waste such as coffee, orange peel, and milk whey [8-10]. However, there is lack of study focusing essential amino acid production from soy pulp since soy pulp has limited content of essential amino acid. Therefore, this study was aimed to enhance essential amino acid in soy pulp.

The aim of the present study is to enhance selected essential amino acid in soy pulp namely tryptophan, lysine, methionine, valine and isoleucine by carbon concentration and fermentation period through solid state fermentation for aquaculture uses.

Materials and Methods

Microorganism

A bacterial strain *Bacillus cereus* was obtained from stock culture of University Malaysia Kelantan, Malaysia. The strain was refreshed and maintained on Tryptone Soy Agar (TSA) agar plates.

Fermentation experiments

Vegetative inoculum was used during fermentation which was prepared by inoculating a loopful of bacteria from the plates into 10 mL of universal bottle containing Tryptone Soy Broth (TSB). The universal bottles were incubated in incubator at 30°C for 24 hrs.

Fermentation media were prepared in a 250 mL of Erlenmeyer flask (soy pulp, 95.5%; yeast extract, 2%; ammonium sulphate, 2%; 0.5% glucose v/v). The hydrolysate was prepared individually by preparing 0.5% (v/v). The media were inoculated with bacterial strain and mixed with hydrolysate thoroughly [11]. The flasks were incubated for 8 days at room temperature (27-30°C) [12]. The fermented soy pulps taken were analysed for the enhancement of tryptophan, lysine, methionine, valine and isoleucine.

Quantification of essential amino acid

The quantitative analysis of isoleucine, valine, methionine, lysine and tryptophan were carried out using acidic ninhydrin method [13,14]. The fermentation products were collected on day 0, 4, 8, and 12 of fermentation to quantify tryptophan, lysine, methionine, valine and isoleucine content. About 5 g of each sample was homogenized with 10 mL of sterilized distilled water and left for 10 mins to obtain the supernatant. The supernatant was filtered using membrane with

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pore size of 0.45 µm. Sample preparations were done in 5 mL capped pyrex tubes. About 50 µL of supernatant from samples were mixed with 550 µL of ninhydrin reagent. The content of Pyrex tubes were heated in 100°C water bath for one hour and let to cool to room temperature. After that, 1600 µL of glacial acetic acid was added into the tubes to dissolve the coloured products. The data of absorbance reading of tryptophan, lysine, methionine, valine and isoleucine were taken at specific wavelength (tryptophan,340 nm; lysine, 470 nm; methionine, 480 nm; valine, 510 nm; and isoleucine, 570 nm) [14-16] using UV Vis Spectrophotometer. The data of absorbance reading of each amino acid was converted into concentration using standard plot of the amino acid. The known concentration of tryptophan, lysine, methionine, valine and isoleucine were prepared using same method as sample preparation to obtain standard plot of concentration versus absorbance reading. In this study, total protein content is not determined.

Statistical analysis

The result of EAA concentration collected from each isolate in this study were analysed by using analysis of two way (ANOVA), with $p<0.05$, followed by Tukey tests using statistical software package Minitab version 17.0.

Results and Discussion

In the present study, fermentation period at day 8 gave the highest production of total essential amino acids (42.9 ± 19.5 gL^{-1}) (Table 1). Decreased production of total essential amino acids was observed after fermentation start and reached the lowest level at day 4 (8.0 ± 2.94 gL^{-1}). However, further fermentation resulted in improved production of total amino acids (42.9 ± 19.5 gL^{-1}) at day 8. After day 8, the production was observed to be decreased (22.4 ± 4.54 gL^{-1}). The decreased in the total essential amino acid production can be explained by the utilization of available free amino acids by the bacteria strain as carbon and nitrogen sources [17].

Sucrose gives the highest and significant effect on total essential amino acid production compared to glucose and molasses with production of (43.1 ± 1.63 gL^{-1}) (Table 2). However, a study carried out the overproduction from genetically engineering *Escherichia coli* reported that there is significant overproduction of amino acid when using glucose as carbon source [18]. In addition, a study of fermentation characterization of *E. coli* also reported that glucose has significant effect towards amino acid production [19]. Furthermore, molasses was reported gave significant effect on amino acid overproduction when using concentration of 10% (w/v) [20]. The contra finding from the present study can be explained as sucrose is completely hydrolized to glucose and sucrose [21] throughout fermentation. Sucrose has the highest and significant effect on lysine production. Similar finding of lysine production by *Corynebacterium glutamicum* was reported which sucrose has higher effect (8%) in producing lysine compared to glucose [22]. This is due to higher secretion of by products with high rate of substrate intake but low production of lysine in TCA cycle from glucose carbon source. Therefore, high rate of input uptake with low rate of output leads to overflow of energy in central metabolism. The lower level of by-products such as lactate can tell the efficiency of carbon flow in central metabolism. When sucrose is supplied as carbon source, it was gradually hydrolysed into glucose and fructose, making it tolerant to glycolysis [23]. In addition, sucrose has high relative osmotic pressure. Usually, glucose is a good carbon source for growth. However, this study reveals that solid state fermentation of soy pulp by *Bacillus cereus* prefer sucrose more than glucose. This was due to tolerance of bacteria to turn on glycolysis instead of TCA cycle and oxidative phosphorylation that would cause accumulation of acetic acid which can reduce cell growth and methionine production [24]. Similar finding was recorded where sucrose give best amino acid production compared to glucose, fructose, maltose and glycerol [25] using. This is due to low accumulation of acetate as by-product in isoleucine production when sucrose is supplemented in fermentation medium compared to other type of carbon sources tested. In addition, the inoculum may have rapid utilization of glucose due to simple structure of sugar compared to other carbon source that will lead to high accumulation of acetate which

Day	Tryptophan (gL^{-1})	Lysine (gL^{-1})	Methionine gL^{-1})	Valine (gL^{-1})	Isoleucine (gL^{-1})	Total (gL^{-1})
0	1.9 ± 0.18	1.4 ± 0.38	7.4 ± 2.36	7.8 ± 1.93	3.6 ± 0.56	22.2 ± 5.05
4	2.4 ± 0.67	0.5 ± 0.12	2.0 ± 0.82	2.6 ± 1.07	0.6 ± 0.96	8.0 ± 2.94
8	1.9 ± 0.01	1.4 ± 0.52	11.8 ± 5.55	17.2 ± 8.39	10.6 ± 5.06	42.9 ± 19.5
12	2.3 ± 0.03	1.0 ± 0.14	6.3 ± 1.23	8.0 ± 1.70	4.8 ± 1.45	22.4 ± 4.54

Table 1: Effect of fermentation period for the production of tryptophan, lysine, methionine, valine and isoleucine.

Day	Tryptophan (gL^{-1})	Lysine (gL^{-1})	Methionine (gL^{-1})	Valine (gL^{-1})	Isoleucine (gL^{-1})	Total (gL^{-1})
Control	1.9 ± 0.07^{a}	0.9 ± 0.01^{bc}	5.2 ± 0.03^{bc}	7.4 ± 0.27^{b}	7.4 ± 0.54^{bc}	22.9 ± 0.33^{bc}
Glucose	2.0 ± 0.03^{a}	0.8 ± 0.04	4.5 ± 0.21	6.5 ± 0.16	5.3 ± 0.39	19.1 ± 0.32
Molasses	2.0 ± 0.04^{a}	1.1 ± 0.09^{ab}	6.7 ± 0.66^{b}	7.1 ± 1.00^{b}	8.6 ± 0.49^{b}	25.5 ± 0.91^{b}
Sucrose	2.1 ± 0.04	1.3 ± 0.08^{a}	9.6 ± 0.60^{a}	16.5 ± 0.94^{a}	13.7 ± 0.92^{a}	43.1 ± 1.63^{a}

Table 2: Effect of different carbon sources for the production of tryptophan, lysine, methionine, valine and isoleucine.

Sucrose concentration (% v/v)	Tryptophan (gL^{-1})	Lysine (gL^{-1})	Methionine (gL^{-1})	Valine (gL^{-1})	Isoleucine (gL^{-1})	Total (gL^{-1})
0	1.9 ± 0.07	0.9 ± 0.01^{a}	5.2 ± 0.03	7.4 ± 0.27	7.4 ± 0.54^{b}	22.9 ± 0.33^{b}
2	2.0 ± 0.03^{a}	1.0 ± 0.04^{a}	6.5 ± 0.61^{a}	8.7 ± 1.07^{a}	7.3 ± 0.92^{a}	43.1 ± 1.63^{a}
4	2.1 ± 0.04	1.3 ± 0.08	9.6 ± 0.60	16.5 ± 0.94	16.7 ± 0.92	21.6 ± 3.45^{b}
6	1.8 ± 0.24	0.9 ± 0.11	5.5 ± 0.91^{ab}	7.2 ± 1.17^{b}	6.2 ± 1.03^{b}	25.8 ± 2.21
8	1.8 ± 0.27^{a}	1.1 ± 0.17	6.5 ± 1.23	8.5 ± 1.50	7.7 ± 1.45	22.8 ± 1.73
10	2.0 ± 0.02^{a}	1.0 ± 0.07^{a}	5.7 ± 0.58^{ab}	7.7 ± 0.98^{b}	6.3 ± 0.72	25.6 ± 2.63

Table 3: Effect of sucrose concentration for the production of tryptophan, lysine, methionine, valine and isoleucine.

is wasting the efficiency of isoleucine central metabolism. Therefore, sucrose which has little complex structure is preferable by bacteria for amino acid production.

The present study found that, total essential amino acid increasing linearly until sucrose concentration reach 2% v/v and declined when sucrose concentration is increased (Table 3). This is because too high concentration of initial carbon source can lead to osmotic stress problems which can lead to carbohydrate transport and carbon flux distribution [19]. Similar finding was reported where close percentage of sucrose (3-5% v/v) gave the highest concentration of amino acid [24]. It was due to decreasing of relative osmotic pressure when cultivation is going on. However, contra finding were reported where the best sucrose concentration for amino acid is 0.9% with isoleucine production of 11.95 gL^{-1} [25] using *E. coli* as inoculum. This is due to difference in osmotic pressure exerted by sucrose to different type of bacteria. Therefore, the optimum osmotic pressure should be further studied for *B. cereus* isolated in this study.

Conclusion

Fermentation period 8 days of soy pulp solid state fermentation was successfully identified in which can enhance total essential amino acid. In addition, the best carbon source for soy pulp solid state fermentation is sucrose with concentration 2% v/v. The finding of this study is important in fish feed formulation for aquaculture use due to more economic source of essential amino acid being used, which is soy pulp compared to fish meal.

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