ISSN : 0974 - 7427 Volume 9 Issue 6

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 9(6), 2015 [252-258]

Medium optimization of neutral protease production by *Bacillus subtilis* ACCC 01746 in solid-state fermentation

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ABSTRACT

The solid state fermentation (SSF) was carried out for the neutral protease production of *Bacillus subtilis* ACCC 01746 (*B. subtilis*). The fermentation factors, which included carbon, nitrogen sources and carbon-nitrogen ratio, and inorganic salts such as Na_2HPO_4 and KH_2PO_4, $ZnCl_2$, $MgCl_2$ and $CaCl_2$, were investigated and optimized when wheat bran was maintained as the only constant carbon source. Maize meal and soybean meal were found best among all the carbon and nitrogen sources tested, respectively, and the ideal maize meal and soybean meal ratio is 4:3. Na_2HPO_4, KH_2PO_4 and $ZnCl_2$ increase the protease production. In contrast, the enzyme activities were inhibited by $CaCl_2$ and $MgCl_2$ When these phosphates were not added in the basal SSF medium. However, $MgCl_2$ and $CaCl_2$ showing marginal increase at low-level concentrations and showing inhabitation at high-level concentrations.

KEYWORDS

Medium optimization;
Bacillus subtilis;
Neutral protease;
Solid-state fermentation.

INTRODUCTION

Bacillus subtilis (B. subtilis) is one of the most investigated microorganisms, because the strain can synthesize and secrete a large number of bioactive substances like extracellular enzymes[1], in which several proteases were secreted by fermentation process[2]. The protease from *B. subtilis* is an important enzyme, has been widely applied and occupied an important position for food industry[3]. Among *Bacillus* proteases, the neutral protease is first isolated and partially characterized by Fukumoto and Negoro[4]. Physical studies show that the neutral protease is zinc metalloenzyme, and stabilized by calcium ions and pH from 6.0 to 10.0. Furthermore, the enzyme can be inactivated by several heavy metals and chelating agents but not by zinc[4].

In past decades, neutral protease has been the object of numerous studies to improve its production methods under submerged fermentation (SBF) or solid-state fermentation (SSF)[5, 6]. Compared with SBF, SSF has highlighted advantages for researchers because of its simple operation, higher product yield and cost-efficiency[7, 8, 9]. Numerous studies

have shown a relationship between the production of neutral protease and physical parameters of SSF[10, 11, 12], in which especially the medium components and varied additives like carbon sources, nitrogen sources and mineral salts greatly influence the enzyme yield[13]. However, there were some problems with the study on the composition of culture medium such as longer generation time and higher production costs. Considering these factors, the objective was to optimize the medium compositions for the cost-effective production of neutral protease by *B. subtilis* ACCC 01746. We used the maize meal, soybean meal and wheat bran to replace the expensive carbon resources like glycerol and sucrose, in the hope of enhancing the production of neutral protease. In addition, the effects of essential inorganic salts such as KH_2PO_4 and Na_2HPO_4, $ZnCl_2$, $MgCl_2$ and $CaCl_2$ need to be included, too.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Bacillus sublitis (ACCC 01746) was received as a present from Dr. Guan (College of Bioengineering, Henan University of Technology) and was cultured on nutrient agar plates (peptone beef extract agar). The plates were incubated at 32 °C for 24 h for the strain until colonies produced. A loopful of the incubated strain was inoculated to 50 ml seed medium (g/L: tryptone 10, yeast extract 5 and NaCl 10; pH 7.0) and incubated in 200 ml conical flask (37 °C, 250 rpm) for 24 h. The cells of *B. subtilis* were then harvested by centrifugation at 8000 rpm, at 4 °C for 15 min and resuspended in sterilized NaCl solution (0.85%) and adjusted to 1×10^8 CFU/mL. The suspension of cells was planning to serve as inoculum for SSF (seed culture).

Preparation of substrates

Different substrates for the SSF viz. carbon sources (maize meal, glycerol, glucose, fructose, malt, sucrose, lactose and corn starch), nitrogen sources (soybean meal, urea, yeast powder, $NaNO_3$, NH_4Cl, tryptone, gelatine and corn syrup), inorganic salts ($MgCl_2$, $ZnCl_2$ and $CaCl_2$) and wheat bran were obtained from a local company in Henan in China (Zhengzhou Jinbaihe Biology Engineering CO., Ltd). These substrates were sterilized at 121 °C and 103 kPa in an autoclave for 20 min if necessary.

Preparation of SSF medium

The basal SSF medium used for neutral protease production, which contained (g): maize meal 40, wheat bran 30, and soybean meal 30; KH_2PO_4 0.3, Na_2HPO_4 1.0 and proper volume of distilled water. Initial pH of the medium was adjusted to 7.0 by 1 mol/L HCl solutions.

The carbon or nitrogen sources of the same weight as a replacement for maize meal or soybean meal in the basal SSF medium were to investigate on their neutral protease production, respectively.

Similarly, the phosphates in basal SSF medium replaced or supplemented further with different salt concentrations ($MgCl_2$, $ZnCl_2$ and $CaCl_2$). According to experimental design, the final concentrations for each additional mineral salt in basal solid media were set to be 0.01%, 0.04%, 0.07%, 0.10%, 0.13%, 0.16% and 0.19%, respectively.

After choosing the best solid substrates, the proportion (2:3, 3:3, 4:3, 5:3, 6:3 and 7:3; w/w) between the chosen carbon and nitrogen sources was further optimized.

The activity of neutral protease in each source study was investigated for predetermined time period at an interval of 6 h. The operating process of the each experiment was replicated three times.

Solid-state fermentation (SSF)

The SSF was conducted in a 250 ml flask that contained different solid dry substrates per bottle. The fermentation flasks were incubated with an inoculum size of 5% seed culture (v/w) and sealed with rubber stopper perforated with a syringe needle for gas release, and incubated at 35 °C for predetermined time period. The samples were taken for analysis of neutral protease at an interval of 6 h of the fermentation.

Analytical methods

The activity of the neutral protease was determined using folin-phenol method according to GB/T23527-2009 of Chinese national standards. 5 g sample (fermented medium) was put into 50 mL of

Regular Paper

phosphate buffer solution (pH7.0) for neutral protease, homogenized on a rotary shaker at 200 rpm for 5 min at 25 ÚC, and then the mixture was filtered by six layers of gauze. The treatment was repeated three times and centrifuged at 12000 rpm, at 4°C for 5 min, and the supernatant was used as enzyme source for the protease assay. One unit of the enzyme was expressed as the amount of enzyme required to release 1 μg of tyrosine by hydrolysates of casein per milliliter and minute in 40°C at pH 7.5 (U/ml), and then converted as total units (U) of neutral protease obtained per gram of dry substrate (U/g).

Based on the maximum enzyme activity, the relative productivity of neutral protease was calculated for each carbon or nitrogen sources.

Statistical analysis

The results of SSF are expressed as mean ± S.D. Analysis of one-way ANOVA and Least Significant Difference (LSD) were used in order to determine the differences among treatments ($P < 0.05$) by SAS (version 9.1).

RESULTS

Effects of carbon and nitrogen sources on the neutral protease activity

The results presented in TABLE 1 and 2 shown that neutral protease activity by *B. sublitis* (ACCC 01746) varied with type of the carbon and nitrogen sources and also dependent on incubation time. When the carbon sources were investigated, wheat bran and soybean meal were maintained as the only constant carbon and nitrogen source. Glycerol showed

TABLE 1 : Effect of different carbon sources on neutral protease production by *B. subtilis* in SSF

Sources	Production [a] (U/g)	SEM	Productivity [b] (U/g·h)	SEM
Glycerol	460 (72 h[c])	12.32	6.39	0.21
Glucose	286 (60 h)	8.34	4.37	0.09
Fructose	350 (60 h)	10.27	5.83	0.24
Malt	368 (54 h)	11.41	6.82	0.17
Sucrose	322 (78 h)	10.26	4.47	0.33
Lactose	450 (72h)	20.34	6.25	0.16
Corn starch	440 (72 h)	15.39	6.11	0.21
Maize meal	349 (48 h)	8.93	7.27	0.08

[1] Values are means of three replicates; [2] SEM: pooled standard error of the means; [3] a: The maximum neutral protease production of different nitrogen sources; b: The maximum neutral protease productivity of different nitrogen sources; c: The fermentation time of maximum neutral protease production.

TABLE 2 : Effect of different nitrogen sources on neutral protease by *B. subtilis* in SSF

Sources	Production[a] (U/g)	SEM	Productivity[b] (U/g·h)	SEM
Urea	201 (36 h[c])	7.24	5.58	0.14
Yeast powder	389 (48 h)	10.05	8.1	0.42
NaNO3	224 (24 h)	9.22	9.33	0.31
NH4Cl	304 (60 h)	11.42	5.07	0.22
Tryptone	307 (48 h)	10.06	6.4	0.09
Soybean meal	462 (48 h)	11.71	9.63	0.41
Gelatin	279 (36 h)	8.88	7.75	0.32
Corn syrup	306 (60 h)	10.23	5.1	0.18

[1] Values are means of three replicates; [2] SEM: pooled standard error of the means; [3] a: The maximum neutral protease production of different nitrogen sources; b: The maximum neutral protease productivity of different nitrogen sources; c: The fermentation time of maximum neutral protease production.

the highest protease activities, followed by lactose, corn starch, malt, fructose and maize meal. Maximum productivity (TABLE 1) was obtained post 48 h of incubation when maize meal was used as the fermentable material. Similarly, the nitrogen sources were chosen on neutral protease production by the strain and maximum the enzyme and the highest productivity were obtained by soybean meal media (TABLE 2). But in the hope of considering the cost-effectiveness for the production of the enzyme, we selected the maize meal to replace the glycerol. Accordingly, maize meal and soybean meal were preferred as the carbon and nitrogen sources in SSF medium for *B. subtilis*.

Effects of inorganic salts on the neutral protease activity

As shown in Figure 1, the basal SSF media with phosphates displayed a higher the enzyme activity compared with the medium without phosphates. Further optimization of the basal SSF medium found that the inorganic salts like $ZnCl_2$ showing positive effects, whereas $MgCl_2$ and $CaCl_2$ showing marginal increase at low-level concentrations and showing negative influence at high-level concentrations (Figure 2 and 3). Accordingly, $ZnCl_2$ was preferred as the inorganic salts sources in SSF medium for *B. subtilis*, whereas $MgCl_2$ and $CaCl_2$ added in media but only as lower levels.

Effects of carbon-nitrogen ratio sources on the neutral protease activity

Further optimization of the media was carried out, and the 4:3 of carbon-nitrogen ratio was the most effective proportion (Figure 4) when wheat bran was maintained as the only constant carbon source. Optimum the enzymatic activity could be achieved when maize meal, wheat bran, soybean meal, KH_2PO_4 and Na_2HPO_4 powders were mixed in a ratio of 40: 30: 30: 0.3: 1. In addition, the optimal levels of $ZnCl_2$, $MgCl_2$ or $CaCl_2$ for neutral protease activity were 0.07% and 0.04% in SSF medium, respectively. When the concentration of $MgCl_2$ or $CaCl_2$ was too high, the enzyme activity significantly decreased.

DISCUSSION

Optimization of different carbon and nitrogen sources

In the present study, wheat bran was chosen as the constant substrate (30.0 g) in basal SSF medium, owing to its carbon sources, mineral-rich salts and porosity that providing a large surface area and helpful in synthesizing and secreting neutral protease for *B. subtilis*[14]. Adesh et al.[15] also found that wheat bran as substrate for the enzyme production was most effective. Similar findings have been reported in different microbial species[16]. Various carbon and nitrogen sources were tested as substrates for neutral

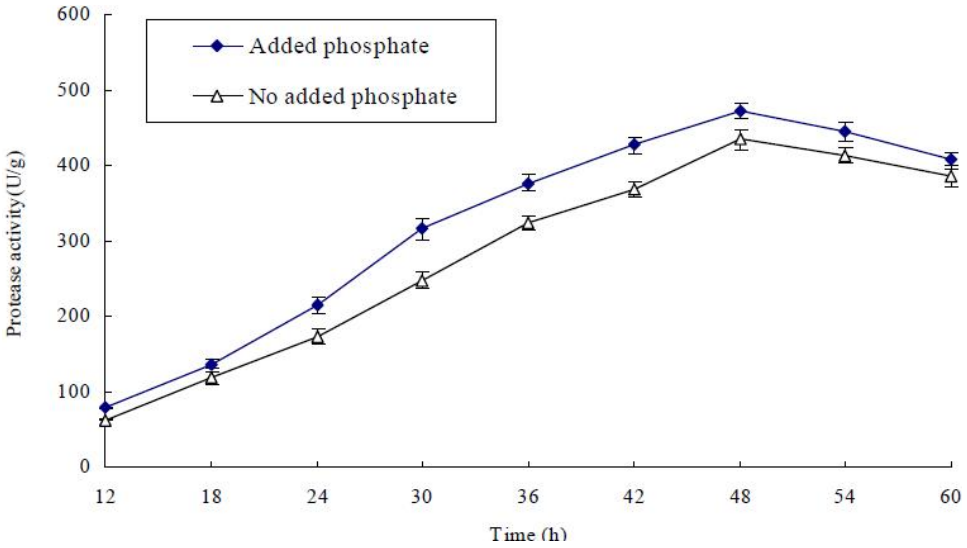

Figure 1 : Neutral protease production by *B. subtilis* ACCC 01746 cultured with added or removed phosphates (N=3), The results are expressed as means ± SEM

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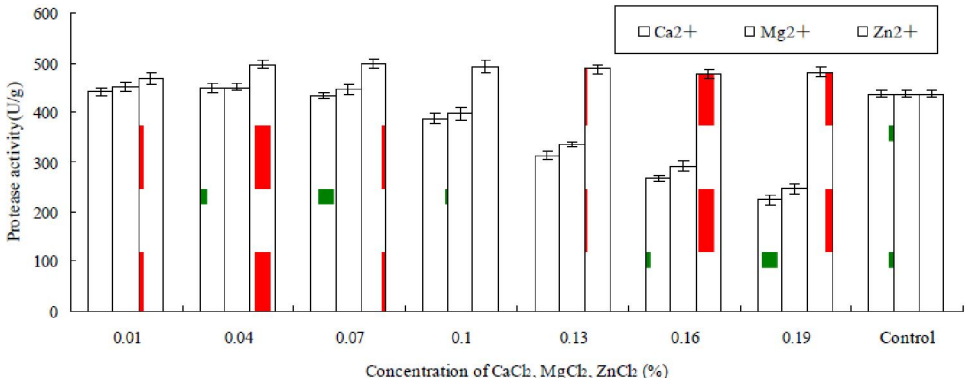

Figure 2 : Neutral protease production by *B. subtilis* ACCC 01746 cultured with different kinds of inorganic salts (added phosphates; N=3), The results are expressed as means ± SEM (three times)

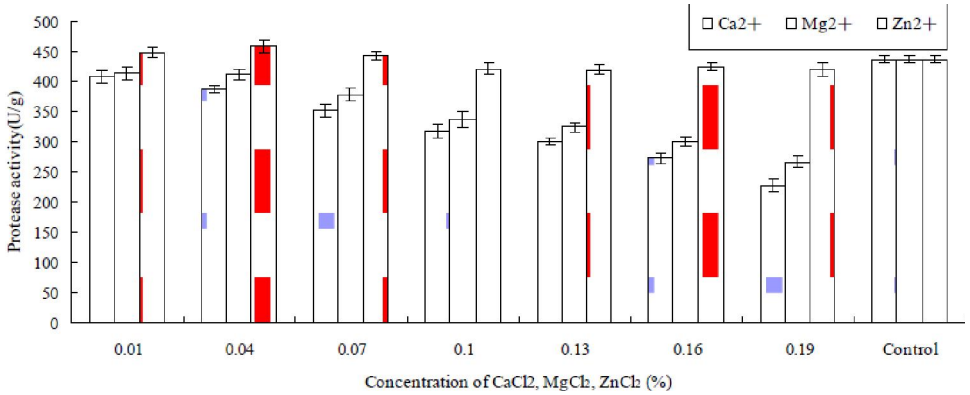

Figure 3 : Neutral protease production by *B. subtilis* ACCC 01746 cultured with different kinds of inorganic salts (removed phosphates; N=3), The results are expressed as means ± SEM (three times)

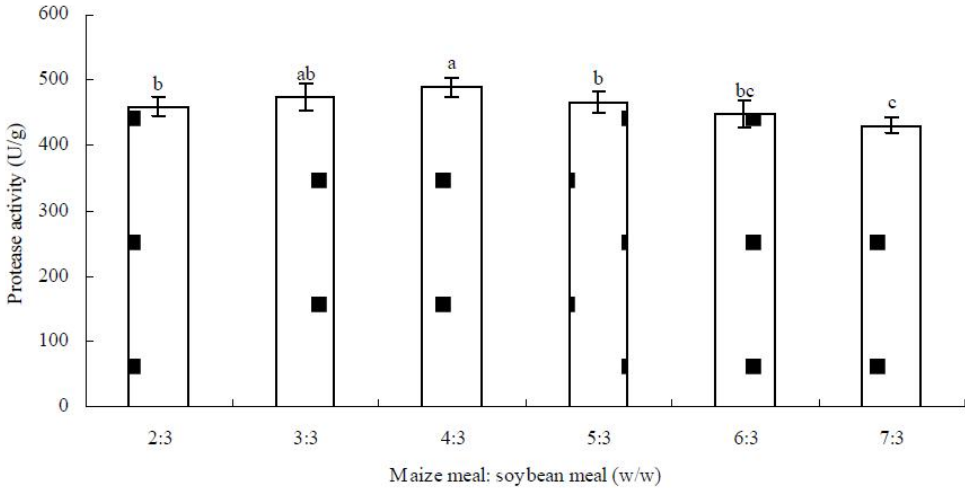

Figure 4 : Neutral protease production by *B. subtilis* ACCC 01746 cultured with different concentrations of carbon-nitrogen ratios (N=3), The results are expressed as means ± SEM

protease activity by *B. subtilis* (ACCC 01746) in SSF and maximum the enzyme activity were obtained at glycerol and soybean meal, whereas the most productivity of carbon sources was maize meal and the nitrogen sources were soybean meal, respectively (TABLE 1). This result can be related to the fact that maize meal with wheat bran and soybean meal in proper proportion. Similar investigation the type and composition of the medium has been reported by Chen et al.[17]. To further select an optimized carbon-nitrogen ratio, six kinds of proportion were investigated. The highest the protease activity was

489.47 U/g as the results shown (Figure 4), while the ratio between maize meal and soybean meal was 4:3 (w/w).

Comparative study on the protease activity by inorganic salts

The strain produced more neutral protease caused by additional KH_2PO_4 and Na_2HPO_4 compared to the production without additional the two salts as the data shown in Figure 1. These phosphates could have positive effect on the protease synthesis of the strain that it might be due to the need for making the cell walls of the bacteria[18]. Therefore, these salts increased production of the enzyme. This result is similar to the neutral protease production by a *B. subtilis* under submerged fermentation condition by using wheat bran as substrates[17]. Further optimization found that the $ZnCl_2$ showed positive effects for the protease, which is most likely due to the enzyme is a zinc-containing enzymes that depend on Zn[19]. The research also found that higher the enzyme biosynthesis at a lower concentration of $CaCl_2$ is most likely due to the protease needed Ca to maintain the stability of the protease[10], whereas the inhibited activity at a higher concentration of $CaCl_2$ or $MgCl_2$ are most probable due to the Zn was replaced by Ca or Mg in the active site of the neutral enzyme, which is similar to the results found by some researchers[10, 17]. In addition, the $HPO4^{2-}$ or H_2PO4^- precipitates Ca^{2+} or Mg^{2+}, which explains the higher enzyme activity observed in 0.04% for $CaCl_2$ or 0.07% for $MgCl_2$ added to the culture medium containing phosphates.

CONCLUSION

The medium compositions for neutral protease production in SSF were optimized in this study. The best carbon and nitrogen sources respectively are corn meal and soybean meal, and the ideal corn meal and soybean meal ratio is 4:3. Phosphates and $ZnCl_2$ increase the protease production. In contrast, $CaCl_2$ and $MgCl_2$ inhibited the enzyme activities when phosphates were not added in the medium. Further optimization found that the $MgCl_2$ and $CaCl_2$ increase the enzyme activity at lower concentrations and inhabit the activity of protease at higher concentrations.

ACKNOWLEDGMENTS

This work was partly supported by the Grant Agreement from Henan Provincial Natural Science Fund Committee (no. 132102110026, China).

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