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Production of a Novel Salt-tolerant L-glutaminase from *Bacillus amyloliquefaciens* Using Agro-industrial Residues and its Application in Chinese Soy Sauce Fermentation

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Abstract: L-glutaminase could hydrolyze L-glutamine to L-glutamic acid which is an important 'Umami' substance. To obtain a salt-tolerant L-glutaminase for application in Chinese soy sauce fermentation, *B. amyloliquefaciens* Y-9 producing salt-tolerant L-glutaminase was isolated from mangrove sediment. The production of L-glutaminase in Solid State Fermentation (SSF) using agro-industrial residues was optimized using a central composite design of the Response Surface Methodology and the enzyme was purified which was used for further characterization including the optimum and stability of pH and temperature, effect of metal ions, substrate specificity and application to soy sauce fermentation. Under optimized conditions the experimental maximum yield of L-glutaminase reached 19.64±0.63 U gds^{-1} which is the highest yield obtained in SSF so far. The L-glutaminase was purified to homogeneity with final specific activity of 196.2 U mg^{-1} protein. The enzyme showed high activity (68% of the original activity) in the presence of 20% NaCl. The enzyme was most stable at pH 5.0 and was highly stable over an acidic pH range (3.0~7.0). These results indicate that this enzyme has a higher salt tolerance and an acidic stability. Furthermore, in Chinese soy sauce fermentation model reaction, the addition of glutaminase from *B. amyloliquefaciens* Y-9 was highly effective for the production of glutamic acid. This study indicates a possibility to establish economical large-scale production of L-glutaminase and this enzyme is suitable for application in soy sauce liquid fermentation with high-salt process.

Key words: L-Glutaminase, optimization, salt-tolerant, soy sauce, *Bacillus amyloliquefaciens*

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is widely distributed in many organisms, including microorganisms (Nandakumar *et al*., 2003) and mammals (Campos-Sandoval *et al*., 2007). It catalyzes the hydrolyic reaction of L-glutamine to L-glutamic acid and ammonia. L-glutaminases are considered to be relevant enzymes for a variety of applications. In food processing industry, the enzyme has been thought to be a useful additive to improve the quality of food in terms of both flavor and nutrition (Wakayama *et al*., 2005). In addition, it has been used for monitoring glutamine levels (Kashyap *et al*., 2002) and for the production of specialty chemicals like thiamine (Nandakumar *et al*., 2003). Although there are many applications of glutaminase, the most studied use of the enzyme has been its ability to hydrolyze L-glutamine to L-glutamic acid which is known to be an important amino acid contributing to a pleasant taste, 'Umami'. In particular, glutaminases have been performed from the standpoint of applications for soy sauce fermentation which occurs under salt concentrations above 15% (Wakayama *et al*., 2005).

In microorganisms, glutaminases have been reported from many species including bacteria, yeasts and fungi (Nandakumar *et al*., 2003). Several microbial enzymes (from *Stenotrophomonas maltophilia* (Wakayama *et al*., 2005), *Micrococcus luteus* (Yoshimune *et al*., 2010), *Rhizobium etli* (Huerta-Saquero *et al*., 2001), *Bacillus pasteurii* (Marcus *et al*., 2002), *Escherichia coli* (Brown *et al*., 2008), *Bacillus subtilis* (Brown *et al*., 2008), *Bacillus licheniformis* (Sinsuwan *et al*., 2012) and *Lactobacillus rhamnosus* (Weingand-Ziade *et al*., 2003) were purified and partially characterized. With respect to the applications for soy sauce fermentation, the interest has been mainly focused in the search of new sources of salt-tolerant glutaminases (Wakayama *et al*., 2005; Weingand-Ziade *et al*., 2003; Iyer and Singhal 2009;

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Yoshimune *et al*., 2004), as well as in the cloning and expression of salt-tolerant enzymes (Wakayama *et al*., 1996; Masuo *et al*., 2005; Ito *et al*., 2011). Wakayama *et al*. (2005) screened various microbes for salt-tolent glutaminase and found one in the *S. maltophilia*. This glutaminase has higher salt-tolerance and thermostability which made it suitable for application in Japanese soy sauce fermentation. A salt-tolerant glutaminase gene *AoglsA*, from *Aspergillus oryzae* RIB40, was expressed in *Saccharomyces cerevisiae* and *E. coli* and the highest expression level was about 186 U mg^{-1} (Masuo *et al*., 2005).

In order to improve growth and enzyme formation of the microorganisms, it is necessary to study the effect of the medium composition and incubation parameters. Recently, Response Surface Methodology (RSM) was employed to optimize glutaminase production by *Providencia* sp., (Iyer and Singhal, 2009) and *Zygosaccharomyces rouxii* (Iyer and Singhal, 2008). These optimization strategies enhanced the enzyme activity and specific activity as compared to the unoptimized media.

For meeting the growing demands and practical applications in the industry, it is necessary to find an organism with highly efficient glutaminase and improve the performance of the system without increasing the cost of production. The enzyme also has unique properties useful for various conditions including its salt-tolerant, temperature stability and wide pH.

In this study, we report the production of extracellular L-glutaminase by halophilic bacteria *Bacillus amyloliquefaciens* Y-9 under SSF conditions. L-glutaminase was purified and characterized subsequently from its culture and application of the enzyme to Chinese soy sauce fermentation process is also discussed. The results demonstrate that Y-9 show good potential as a glutaminase producer. And L-glutaminase from strain Y-9 has been considered to be useful in Chinese soy sauce fermentation.

MATERIALS AND METHODS

Microorganism and inoculums: The mangrove sediment samples collected from different places along the beach of Guangdong of China were used as the isolation source of solt-tolerant glutaminase-producing microorganisms. A certain amount of each of samples was suspended in sterilized water and spread on isolation medium agar plates (glutamine 5 g, $NaHPO_4$ 5 g, KH_2PO_4 3 g, NaCl 50 g, $MgSO_4.7H_2O$ 0.1 g, $CaCl_2$ 0.2 g, agar 15 g, sterilized water 1000 mL and pH 6.0~7.0). After incubation at 30°C for 2~3 days, colonies grown on the plate were isolated. And each isolate was incubated at 30°C for 3~4 days with shaking, following which supernatant were harvested and the glutaminase activity of each isolate was measured according to the assay method described below. The strain Y-9 was selected from 650 isolates grown on isolation medium.

Inocula of Y-9 were prepared in 500 mL Erlenmeyer flasks containing 100 mL of liquid medium (glucose 10 g, yeast extract 5 g, $NaHPO_4$ 6 g, KH_2PO_4 2 g, NaCl 8 g, sterilized water 1000 mL and pH 6.5). The inoculated strain was kept in a shaker at 200 rpm for 48 h and used as the inoculum. This inoculum (5×10^7 cells mL^{-1}) was used for all subsequent inoculations unless otherwise mentioned.

Chemicals and solid substrates: Glutamine, asparagines and γ-glutamyl-p-nitroanilide were purchased from Sigma chemicals (China). Solid substrate, namely agro-industrial residues wheat bran, rice bran, bean dregs, groundnut cake, sugarcane bagasse, wood shavings were obtained locally.

Solid-state fermentation (SSF): About 500~1000 g of media containing (g kg^{-1}) in enamel coated metallic trays (120×100×10 cm) was used to produce L-glutaminase with SSF. The medium compositions were based on the single-factor experiments and RSM experimental design. The initial pH and moisture content of the substrates were adjusted by adding HCl or NaOH, respectively and then autoclaved at 121°C for 20 min. The trays were cooled to room temperature and each was inoculated with inoculum prepared as mentioned earlier containing 5×10^7 cells mL^{-1}. They were then incubated in a stationary mode with a relative humidity of 85% for 48 h. All fermentation experiments were done in triplicate.

Experimental design and fermentation optimization: After optimizing the nutrients by primary screening using a one-variable-at-a-time approach, RSM was applied using CCD for optimizing the L-glutaminase production of strain Y-9. The wheat bran (X_1), soybean cake (X_2) and tap water (X_3) were chosen as the independent variables and L-glutaminase (Y) was used as dependent output variables. Values of production of L-glutaminase were taken as the responses of the design experiment. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). All the experiments were done in triplicate and 'Design-Expert' software (version 8.0.7, Stat-Ease Inc., Minneapolis, USA) was used for the regression analysis and the graphical presentation (Masui *et al*., 2012; Suresh, 2012).

Enzyme activity: Enzyme activity was assayed according to the method of Moriguchi *et al*. (1994). The reaction

mixture, containing 1.8 mL of 0.1 M acetic acid buffer (pH 6.5), 0.1 mL of 50 mM L-glutamine and 0.1 mL of crude enzyme extract was incubated at 50°C for 20 min. The reaction was terminated by adding 0.5 mL 3 M HCl and kept at 4°C for 5 min later 100 mM Tris-HCl buffer (pH 9.4), 20 mM β-NAD, 0.5 mM ADP and 20 U mL^{-1} of L-glutamic dehydrogenase were added in a total volume of 285-15 μL of reaction mixture from the earlier step. The reaction was conducted in UV transparent 96-well plates and read on a Microplate Reader (Thermo Fisher Scientific Inc, USA). The absorbance at 340 nm was measured before and after incubating the mixture for 30 min at 37°C. One unit of L-glutaminase activity was defined as the amount of enzyme that liberated 1.0 μmol of glutamine under optimal assay conditions. Enzyme yield was expressed as units per gram dry substrate (U gds^{-1}).

The protein concentration was determined by the Folin Lowry method using bovine serum albumin as the standard (Lowry *et al*., 1951). Each sample was tested in triplicate.

Enzyme extraction and purification: After incubation, the enzyme extraction was carried out by mixing the solid media with 0.1 M phosphate buffer (pH 7.0) for 1 h on water baths at 45°C. And the contents were separated from the inert support by filtering through a cheese cloth. The separated solution was then centrifuged at 12000×g, 4°C and 10 min for removing the microbes. The supernatant obtained was used for enzyme purification.

Purification of L-glutaminase was done by the method proposed by Wakayama *et al*. (2005). The supernatant was fractionated by ammonium sulfate precipitation and dialyzed in 10 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was then applied to a DEAE-Sepharose FF anionic exchange chromatography column (25×1.6 cm; Pharmacia). After washing the column with Tris-HCl buffer (pH 8.0), active fractions were obtained by elution with a linear salt gradient (0~0.2 MNaCl in 10 mM Tris-HCl buffer, pH 10.0). The main L-glutaminase containing fractions were dialyzed against Tris-HCl buffer (pH 8.0), loaded onto a hydroxyapatite column (20×1.0 cm) and eluted with a linear gradient of 5~100 mM potassium phosphate buffer (pH 7.0). The enzyme, concentrated by ultrafiltration (Amicon), was applied to a Sephacryl S-200 gel filtration column (15×1.5 cm, Pharmacia) and run with Tris-HCl buffer (pH 8.0) supplemented with 100 mM NaCl. The active fractions were collected and dialyzed against Tris-HCl buffer (pH 8.0). The enzyme preparation was concentrated by ultrafiltration and stored at 4°C.

The purity and molecular mass of the purified L-glutaminase was estimated with SDS-PAGE using a 5% stacking gel and a 12.5% resolving gel. This was followed by staining with Co-omassie brilliant blue R-250.

Substrate specificity: The specificity of the purified enzyme against different substrates was investigated. Alternative substrates for measurement of L-glutaminase activity were L-glutamine (L-Gln), D-glutamine (D-Gln), L-asparagine (L-Asn), D-asparagines (D-Asn) and γ-glutamyl-p-nitroanilide (γ-GNP). As described by Imada *et al*. (1973) with slight modifications, the assay mixture for glutaminase activity contained 0.1 M acetic acid buffer (pH 6.5), different substrates and purified enzyme in a final volume of 2.0 mL. After a 20 min reaction at 60°C, the reaction was stopped by boiling for 5 min and centrifuged at 12000 rpm for 5 min. The concentration of glutamic acid or arginine of reaction mixture was measured with a High Performance Liquid Chromatography (HPLC) system (Kijima and Suzuki, 2007). While using γ-GNP as substrate, 2 mL of 1.5 M acetic acid was added to stop the enzyme reaction and then the absorbance was measured at 410 nm (Thammarongtham *et al*., 2001).

Effect of temperature on L-glutaminase activity and thermostability: The temperature optimum was measured by performing the L-glutaminase activity assay at various temperatures under pH 6.5 using acetic acid buffer. The thermostability of the L-glutaminase was investigated by pre-incubation of the enzyme solutions for 60 min in the absence of substrate in acetic acid buffer (pH 6.5), at temperatures 40, 45, 50, 55, 60, 65, 70 and 80°C, respectively. Residual activities were determined under L-glutaminase activity assay conditions.

Effect of pH on L-glutaminase activity and stability: The effect of pH on this enzyme was evaluated at the optimal temperature (60°C) over a pH range of 3.0-12.0, using 0.1 M borate-citrate-phosphate buffer. Further study on the pH stability was carried out by pre-incubating the enzyme solutions at 4°C for 1 h in the aforementioned buffer systems in the absence of substrate. The pH values of various reaction solutions were adjusted to pH 7.5 and then they were subjected to L-glutaminase activity assay.

Effect of inhibitors and metal ions on enzymatic activity: After pre-incubating the enzyme solutions containing each individual inhibitors and metal ions at pH 6.5 and 0.1 M acetic acid buffer at 4°C for 15 min, substrate glutamine was then added and the enzyme activity was measured as described above under standard conditions. A control without inhibitors and metal ion was also performed. The amount of enzymatic activity measured

was calculated as a percentage of the activity comparing to that of the control. The final concentrations of the tested inhibitors and metal ions were 0.1 and 1 mM.

Effect of NaCl on L-glutaminase activity and stability: The effect of NaCl on purified L-glutaminase was studied by adding 0-20% (w/v) NaCl to the reaction mixture. The stability at different concentration of NaCl was studied by incubating the purified enzyme at 18% NaCl and measuring the remaining activity was assayed periodically in the standard assay.

Estimating glutamic acid production by L-glutaminase: The effect of glutaminase on glutamic acid production was performed according to the conditions described previously (Wakayama *et al*., 2005) with some modifications. In brief, a reaction system (100 mL) consisted of 10 g soybean protein, 15 or 18 g NaCl, the crude protease obtained from Koji culture and a certain amount of glutaminase from the solid media (final NaCl concentration; 15 or 18%). The reaction was carried out at 30°C for 60 h and then the concentrations of glutamic acid in reaction mixture were measured with HPLC system as described previously.

RESULTS AND DISCUSSION

Strain identification: Strain Y-9, exhibiting relatively high L-glutaminase activity, was isolated from mangrove sediment of Guangdong. It was an aerobic, Gram-positive bacterium that could grow at 50°C. The biochemical characteristics of the bacterium were as follows: spores positive, oxidase positive, catalase positive, reduced nitrate positive, Voges-Proskauer positive, methyl red test negative, citrate positive, casein hydrolysis positive, starch hydrolysis positive. Strain Y-9 could grow on D-glucose, arabinose, sucrose, D-xylose, mannitol, lactose and D-fructose as carbon sources. A DNA fragment amplified from the genomic DNA of strain Y-9 with primers (16F: 5'-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3'and 16R: 5'-TAC GGC TAC CTT GTT ACG ACT CAC CCC-3') corresponding to 16S rDNA consensus sequences was directly sequenced. The nucleotide sequence determined, 1480 bp, was analyzed by BLAST and strain Y-9 was classified as *Bacillus amyloliquefaciens* (identity: 96%, GenBank accession number: EU855194.1). As the results, we designated strain Y-9 as *B. amyloliquefaciens* Y-9.

Single-factor experiments: Compared to the control, all carbon sources added to the SSF substrates improved L-glutaminase production. Maximum L-glutaminase production (9.2 U gds^{-1}) was obtained when wheat bran was the supplement, at a level of 25% (w/w), followed by wood shavings (7.4 U gds^{-1}), sugarcane bagasse (6.6 U gds^{-1}) and rice bran (6.4 U gds^{-1}). The results with wheat bran were similar to that of (Kashyap *et al*., 2002). For nitrogen sources, soybean cake resulted in maximum L-glutaminase production (10.6 U gds^{-1}), at a level of 25% (w/w), followed by corn flour (9.4 U gds^{-1}), groundnut cake (7.8 U gds^{-1}) and rapeseed cake (5.4 U gds^{-1}). To study influence of mineral salt additions on L-glutaminase biosynthesis, the media were supplemented with some mineral salt NaCl, K_2HPO_3, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, $FeCl_3.H_2O$, $CuSO_4$ and $CaCl_2$ to final concentrations of 8%, 0.5, 0.5, 0.2, 0.2, 0.3 and 0.2% (w/w), respectively. Our date showed a positive effect of the addition of NaCl, K_2HPO_3, $MgSO_4.7H_2O$ and $CuSO_4$ on L-glutaminase production (40, 35, 10 and 13% compared to the control, respectively). However, $MnSO_4.H_2O$, $FeCl_3.H_2O$ and $CaCl_2$ supplemented to the medium did not enhance L-glutaminase production significantly likely because theses elements were already present in saturating amounts in the other materials.

With the increase in inoculum size, there was a gradual increase in the production of enzyme and maximum L-glutaminase (11.2 U gds^{-1}) was produced when 2% inoculum was used. Higher levels of inoculation resulted in decreased yields. Maximum production L-glutaminase of 11.8 U gds^{-1} was achieved at 30°C. This observation is consistent with the results of (Kashyap *et al*., 2002) which were a decline in the enzyme titers and the pattern was more or less similar for both substrates (El-Sayed, 2009). L-glutaminase production increased along with an increase in moisture levels to a maximum of 12.3 U gds^{-1} at 50%. But higher moisture levels reduce yield due to substrate particle agglomeration causing oxygen transfer difficulty (Pandey, 1994; Sudhir *et al*., 2009). Maximum production (12.8 U gds^{-1}) of L-glutaminase was obtained at an initial pH of 7.0. Previous studies of L-gultaminase under SSF have shown an initial moisture level of 60~80% and pH 6.0-9.0 to be optimal (Kashyap *et al*., 2002; Sabu *et al*., 2000). These differences might be due to the differences in the experimental substrates and bacterial species.

Optimization by response surface methodology: Based on above results of single-factor experiments, the importance of nine variables, viz., wood shavings, wheat bran, soybean cake, corn flour, NaCl, $CuSO_4$, K_2HPO_3, inoculums and tap water, for L-glutaminase production was investigated by PBD. By the statistical analysis, the factors having the greatest impacts on the production of L-glutaminase were wheat bran, soybean cake and tap water (data no show).

The Central Composite Design (CCD) was further employed to study the interactive effects of wheat bran, soybean cake and tap water (newly designated as X_1, X_2 and X_3, respectively) and also to determine their optimal levels on the production of L-glutaminase. Twenty experiments including three center points were conducted with three independent variables at five levels (-1.68, -1.00, 0.00, +1.00 and +1.68). The experimental response is represented in Table 1. The second-order polynomial equation on the L-glutaminase production was derived, as follows:

$$Y = 17.368+0.040X_1+0.108X_2+0.628X_3-0.772X^2_1-0.352X^2_2-1.14X^2_3-0.104X_1X_2-0.281X_1X^2-0.005X_2X_3 \quad (1)$$

where, Y is the predicted L-glutaminase production, X_1 is wheat bran, X_2 is soybean cake and X_3 is tap water.

The ANOVA of the quadratic regression model suggested that Eq. 1 reflected a highly significant model which was evident from the 'F-value' (102.45) and 'p-value' (<0.0001). The model also showed a statistically nonsignificant lack-of-fit (p = 0.065), so the model was supposed to be adequate for prediction within the range of variables employed. The coefficient of determination (R^2) which expressed the quality of fit for the polynomial model equation, was 98.88%. Moreover, the Adj R^2 (93.82%) also confirmed a high significance of the model. Moreover, the low value of the coefficient of variation (CV = 1.20%) indicated that the experiments had a good precision and reliability.

The interaction effects and optimal levels of the variables were determined by examining the planned series of three-dimensional (3D) response surface plots obtained using Design-Expert software. These plots were generated for the pair-wise combination of the three variables while keeping the third at 'zero' levels. Figure 1a represents the interaction between wheat bran and soybean cake while keeping tap water at a zero level. The results demonstrated that with the increase of wheat bran and soybean cake to 175.1 and 201.5 g, respectively, L-glutaminase production increased to 17.38 U gds^{-1}. After that, lower and higher levels of the concentration of wheat bran and soybean cake did not result in higher yields which was in agreement with the model. The interactive effect of wheat bran and tap water was demonstrated in Fig. 1b, where soybean cake was kept at a zero level. The L-glutaminase production was found to increase with the reduction at wheat bran and increase in tap water and decreased thereafter. Figure 1c expresses the interaction between soybean cake and tap water which showed that simultaneous increase in both the factors contributed to the maximum L-glutaminase production of 17.48 U gds^{-1}, beyond which a decrease in L-glutaminase production was observed.

The model predicted a maximum L-glutaminase production of 17.48 U gds^{-1} after 48 h incubation in the optimum fermentation medium containing 174.8 g wheat bran, 50 g wood shaving, 201.6 g soybean cake, 25 g corn flour, 353.0 g tap water and with inoculum concentration of 2.0%, incubation temperature of 30°C and initial pH of pH 7.0, respectively.

To confirm the predicted results and the model adequacy, five additional validation experiments in metallic trays containing 1000 g of SSF medium with the

Table 1: Central composite experiment design with the three significant variables and its response

	Coded levels			L-glutaminase production (U gds^{-1})	
Runs	X_1 (g)	X_2 (g)	X_3 (g)	Actual	Predicted
1	170 (-1.00)	190 (-1.00)	360 (1.00)	15.72±0.53	15.82
2	175 (0.00)	200 (0.00)	350 (0.00)	17.26±0.51	17.37
3	175 (0.00)	200 (0.00)	350 (0.00)	17.42±0.56	17.37
4	175 (0.00)	200 (0.00)	376.8 (1.68)	15.48±0.62	15.29
5	180 (1.00)	210 (1.00)	340 (-1.00)	14.66±0.44	14.75
6	175 (0.00)	200 (0.00)	350 (0.00)	17.50±0.49	17.37
7	175 (0.00)	216.8 (1.68)	350 (0.00)	16.75±0.52	15.26
8	175 (0.00)	200 (0.00)	323.2 (-1.68)	13.08±0.65	13.00
9	166.6 (-1.68)	200 (0.00)	350 (0.00)	15.13±0.51	16.19
10	170 (-1.00)	210 (1.00)	340 (-1.00)	14.22±0.58	14.73
11	175 (0.00)	183.2 (-1.68)	350 (0.00)	16.27±0.44	15.12
12	170 (-1.00)	210 (1.00)	360 (1.00)	16.20±0.52	15.54
13	180 (1.00)	210 (1.00)	360 (1.00)	15.29±0.45	15.54
14	180 (1.00)	190 (-1.00)	360 (1.00)	15.44±0.41	16.23
15	180 (1.00)	190 (-1.00)	340 (-1.00)	14.58±0.53	14.31
16	183.4 (1.68)	200 (0.00)	350 (0.00)	15.52±0.60	16.55
17	175 (0.00)	200 (0.00)	350 (0.00)	17.22±0.53	17.37
18	170 (-1.00)	190 (-1.00)	340 (-1.00)	13.94±0.54	13.88
19	175 (0.00)	200 (0.00)	350 (0.00)	17.46±0.42	17.37
20	175 (0.00)	200 (0.00)	350 (0.00)	17.30±0.51	17.37

X_1: Wheat bran, X_2: Soybean cake and X_3: Tap water

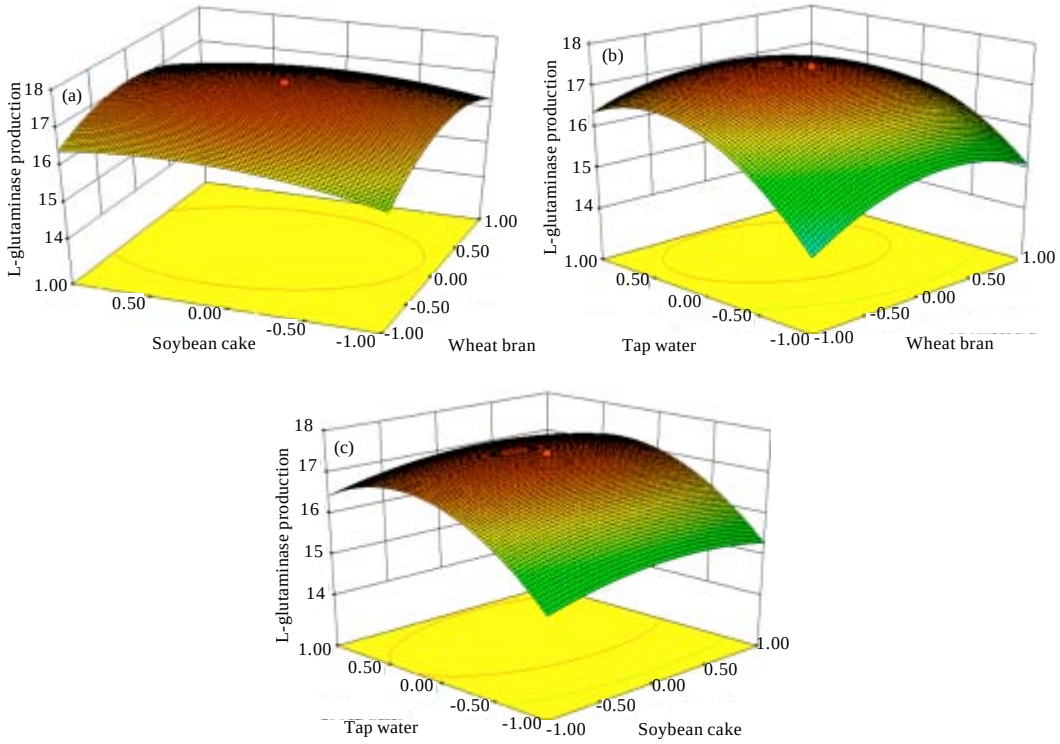

Fig. 1(a-c): Three-dimensional (3 D) response surface plots for L-glutaminase production by *B. amyloliquefaciens* Y-9 showing variable interactions of (a) Wheat bran and soybean cake, (b) Wheat bran and tap water and (c) Soybean cake and tap water

Table 2: Summary of purification of the L-glutaminase produced by Y-9

	Total protein (mg)	Specific activity (U mg^{-1})	Total activity (U)	Yield (%)	Purification (fold)
Supernatant	912.0	1.92	1748	100	1.0
DEAE-Sepharose FF	36.0	25.70	926	53	13.4
Hydroxyapatite	4.2	93.70	384	22	48.9
Sephacryl S-200	0.8	196.20	157	9	102.4

optimum compositions were performed. The actual average L-glutaminase production was 19.64±0.63 U gds^{-1} which agreed well with the predicted value, indicating that the suggested model was valid for predicting L-glutaminase production.

Although, Submerged Fermentation (SMF) have produced impressive 119~155 U L^{-1} L-glutaminase (Iyer and Singhal, 2008, 2009), the relatively high costs of media constituents in SMF make it an expensive process. SSF could offer an opportunity to reduce energy requirements and costs of media in the production of L-glutaminase. In the present study, agro-industrial residual, such as wheat bran and soybean cake, were selected as the SSF substrates. By the optimized condition, we obtained the maximum L-glutaminase production of 19.64 U gds^{-1} which was higher than the results obtained by other investigators (Sabu *et al*., 2000; Kashyap *et al*., 2002; Singh and Banik, 2012).

Purification of L-glutaminase: The enzyme purification result has been summarized in Table 2. The enzyme was purified to the overall yield from the crude extract of about 9%, with a purification of 102.4 fold and a specific activity of 196.2 U mg^{-1} proteins. The purified enzyme showed a single band by SDS-PAGE analysis (Fig. 2) which suggests that the glutaminase was purified to homogeneity. And SDS-PAGE analysis also revealed the molecular mass of purified glutaminase was approximately 35 kDa. In general, most glutaminase have been shown have typical molecular masses of 30~50 kDa (Nandakumar *et al*., 2003; Wakayama *et al*., 2005). While

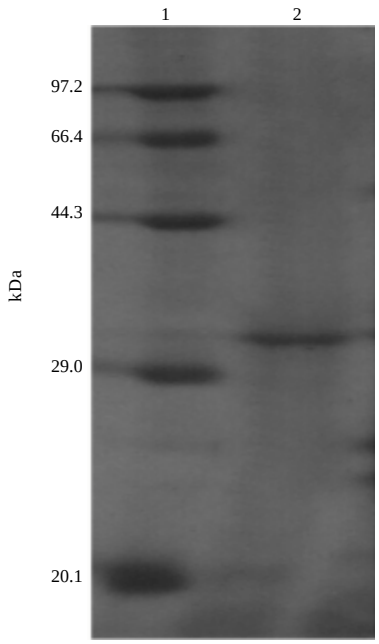

Fig. 2: SDS-PAGE analysis of purified L-glutaminase from Y-9, Lane 1: Standard protein molecular mass marker (TaKaRa, sizes in kilodalton are indicated on the right) and Lane 2: Purified glutaminase protein

Table 3: Substrate specificity of L-glutaminase from Y-9

Substrate	Relative activity (%)
L-glutamine	100
D-glutamine	24
L-asparagine	93
D-asparagine	11
γ-glutamyl-p-nitroanilide	102

the purified extracellular glutaminase YbgJ and YblM of *B. subtilis* is a 36.2 and 34.0 kDa, respectively (Brown *et al*., 2008), the glutaminase of *M. luteus* K-3 is of 48.3 kDa (Yoshimune *et al*., 2010).

Substrate specificity of purified enzyme: The substrate specificity of L-glutaminase from Y-9 was determined using several glutamyl derivatives. As shown in Table 3, L-glutamine and γ-glutamyl-p-nitroanilide were the best substrate for the enzyme and it also catalyzed the hydrolysis of L-asparagine as good substrates (93%). However, activity for D-glutamine and D-asparagine were only 24 and 11%, respectively of that for L-glutamine. These findings suggests that the enzyme from Y-9 could hydrolyzes L-, D-glutamine and L-, D-asparagine, but not be classified as conventional glutaminase asparaginase (E.C. 3.5.1.38), as it hydrolyzes L-glutamine and L-asparagine other than D-glutamine and D-asparagine

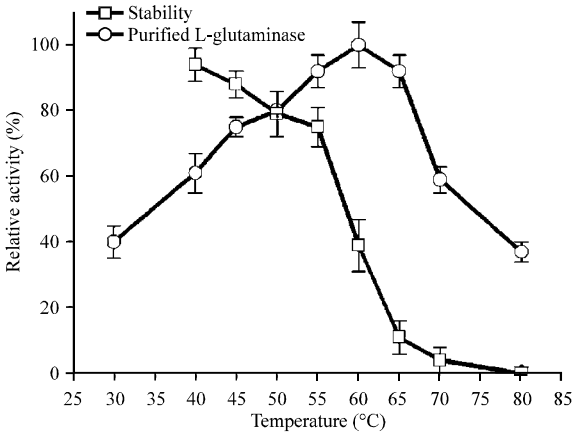

Fig. 3: Effect of temperature on activity, Data points are the average of triplicate measurements, error bars represent±1 SD

(Sato *et al*., 1999). The enzyme exhibits the highest activity against L-conformation substrates which maybe expand its application areas such as medicine, for which some actions need space specificity of compounds. In addition, the L-glutaminase from Y-9 differed from bacterial glutaminase in its activity for γ-glutamyl-p-nitroanilide as well as L-glutamine (Nandakumar *et al*., 2003; Wakayama *et al*., 2005).

Effect of temperature on catalytic activity and stability: The optimal temperature for the enzyme was 60°C and the enzyme was stable under 55°C (Fig. 3). The glutaminase retained 40% activity even after treatment at 60°C, but the enzyme was drastically reduced by incubation at 70°C for 60 min (Fig. 3). As far as the temperature dependence of glutaminases is concerned, many of the glutaminases reported have both an optimal and stable temperature of around 40~50°C (Nandakumar *et al*., 2003). However, Glutaminase from *S. maltophilia* NYW-81 retain 50% residual activity even after heat treatment at 70°C for 10 min (Wakayama *et al*., 2005).

Effect of pH on catalytic activity and stability: To determine the optimal pH for the L-glutaminase, we measured the enzyme activity at various pH-values (pH 3.0-12.0). The pH-activity profile of the enzyme was bell-shaped. The pH value for optimal activity was determined to be 6.5, with about 50% of maximum activity being retained in pH 4.0 (Fig. 4). Moreover, the enzyme was found to be stable in the pH range of 3.0-8.0 and more than 60% of the activity was remained (Fig. 4), confirming that L-glutaminase from Y-9 has broader an acidic pH activity range. Generally, most microbial glutaminase have an alkaline pH optimum at pH 7.0~9.0, (Nandakumar *et al*.,

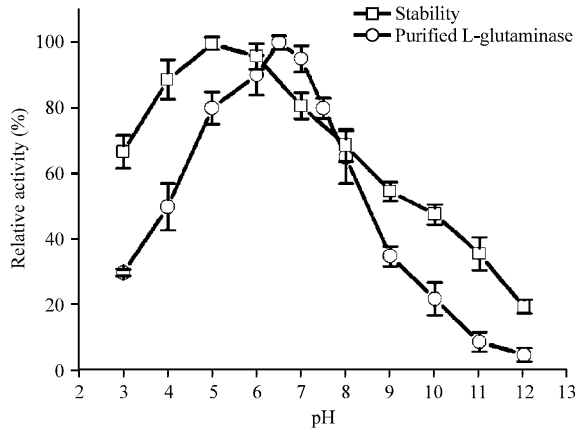

Fig. 4: Effect of pH on activity, Data points are the average of triplicate measurements, error bars represent±1 SD

Table 4: Effect of metal ions and inhibitors on the activity of purified L-glutamianse

		Relative activity (%)	
Inhibitprs	Metal ions	0.1 (mM)	1 (mM)
None	None	100	100
EDTA		90	80
DTT		85	0
NaN_3		50	0
	Mn^{2+}	85	80
	Mg^{2+}	105	100
	Co^{2+}	100	90
	Zn^{2+}	100	96
	Fe^{2+}	98	92
	Ni^{+}	100	100
	Ca^{2+}	102	92
	K^{+}	100	110
	Al^{3+}	78	46

2003; Weingand-Ziade *et al*., 2003; Wakayama *et al*., 2005). Recently, an acidic glutaminase (YbaS) from *E. coli* was found (Brown *et al*., 2008). This enzyme expressed maximal activity even at pH 4.5 with L-glutamine as the substrate. However, the pH stability of the enzyme was not reported. In this study, L-glutaminase from Y-9 not only showed an acidic pH optimum for L- glutamine substrates (6.5), but also is resistant to the acidic pH range of 3.0~7.0. These results indicate that L-glutaminase from Y-9 is an acidic glutaminase. And this property underlines its potential in the soy sauce fermentation process, the main fermentation within the pH range 5~6 (Wakayama *et al*., 2005), for which most microbial glutaminase are unsuitable.

Effect of inhibitors and metal ions on enzyme activity: The effect of different inhibitors and metal ions on the purified L-glutamianse activity was investigated (Table 4). The results revealed that some inhibitors and metal ions imparted distinct effect on the enzymatic activity. When added at low concentrations (0.1 mM), Ca^{2+} and Mg^{2+} increased the enzymatic activity while EDTA, DTT, Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, Ni^{+} and K^{+} barely or slightly decreased the enzymatic activity and NaN_3 and Al^{3+} decreased the enzymatic activity remarkably. When added at high concentration (1 mM), K^{+} enhanced the enzyme activity markedly and the enzyme activity reached up to 1.1 fold of original that. Moreover, 1 mM of Mg^{2+} and Ni^{+} had no negative effects the enzymatic activity and EDTA, Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} and Ca^{2+} inhibited the enzyme activity partially. However, the enzymatic activity was completely inhibited in the presence of 1 mM of DTT and NaN_3.

Effect of NaCl on catalytic activity and stability: The effect of NaCl concentration on the activity of the purified glutaminase was determined by adding 0~25% (w/v) NaCl to the reaction medium. The glutaminase could tolerate higher concentrations up to 18% NaCl (19% loss in activity), even in the presence of 20% NaCl, the enzyme still retained 68% of its activity compared to the reaction without NaCl (Fig. 5a). Similarly, the purified enzyme could tolerate up to 18% NaCl for 30 days with only 45% loss in activity at 25°C (Fig. 5b). Thus, it could be concluded that glutaminase from Y-9 is halotolerant. Glutaminase from various microorganisms have differently in the presence of NaCl. The salt tolerance of the enzyme was similar to that of the enzymes from *M. luteus* and *B. subtilis* (100 and 94% activity, respectively, in the presence of 16% NaCl) which were the most halotolerant at present (Nandakumar *et al*., 2003; Weingand-Ziade *et al*., 2003). However, the residual activity of their enzyme in the presence of NaCl over days was not reported. The glutaminase from Y-9 could retain 55% of its activity for 30 days with up to 18% NaCl at 25°C which suggests that the enzyme works adequately under the NaCl conditions where Chinese soy sauce fermentation is carried out.

Effect of purified L-glutaminase supplementation on glutamic acid production in a model reaction of soy sauce fermentation: It is well known that glutaminase is an important additive during soy sauce fermentation, because of responsible for the synthesis of flavor-enhancing amino acid, glutamic acid (Nandakumar *et al*., 2003). So that, the effect of L-glutaminase from Y-9 on glutamic acid production in a model reaction of Chinese soy sauce fermentation was investigated. Results of the glutamic acid produced are shown in Fig. 6. The glutamic acid production of reaction mixture was higher with the addition of purified L-glutaminase from Y-9 (1.0 U g^{-1}) than without. The glutamic acid concentration of mixture with glutaminase was no less than 1.75 g L^{-1} higher than that without the

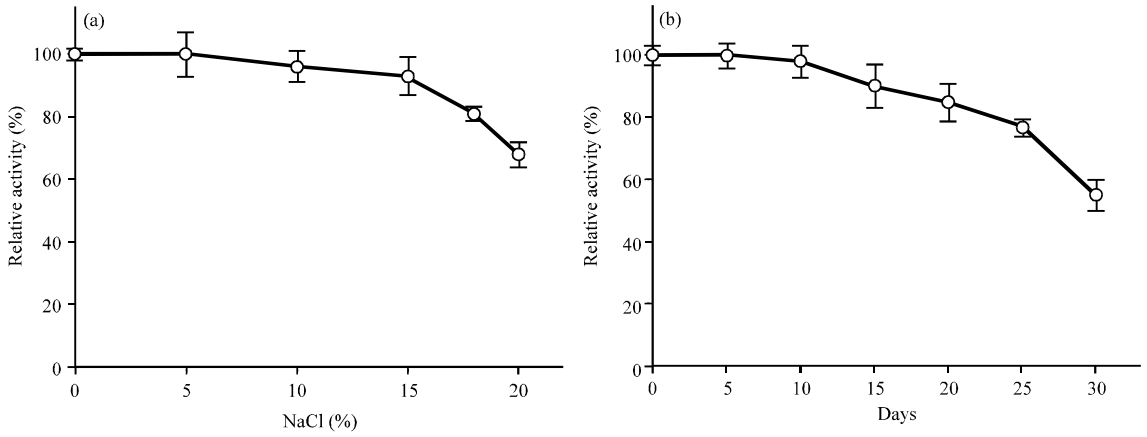

Fig. 5(a-b): (a) Effect of NaCl concentration on glutaminase activity and (b) Residual activity of glutaminase in the presence of 18% NaCl over 30 days at 25°C, Data points are the average of triplicate measurements, error bars represent±1 SD

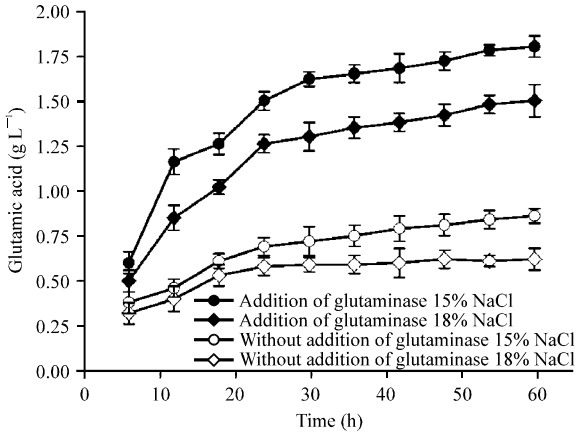

Fig. 6: Effect of purified glutaminase from *B. amyloliquefaciens* Y-9 on glutamic acid production

addition of glutaminase at the end of reaction. From these results, the addition of L-glutaminase from Y-9 could speed up the production of glutamic acid. Moreover, compared with glutaminases from other reported microbial sources, the addition of glutaminase from Y-9 was most effective for the production of glutamic acid, even if in 18% NaCl (Wakayama *et al*., 2005). The results indicate that L-glutaminase from Y-9 has significant advantages over other microbial glutaminases in the production of glutamic acid in soy sauce fermentation processes.

CONCLUSION

A novel L-glutaminase was obtained by strain isolation. The isolated *B. amyloliquefaciens* Y-9 secreted a high level of L-glutaminase under SSF using agro-industrial by-products within a relatively short period of incubation. Furthermore, the L-glutaminase was purified and characterized. Results showed that its properties of highly salt-tolerant, acidic activity and substrate specificity were distinct from other microbial glutaminase reported before. These unusual properties make L-glutaminase from Y-9 an interesting enzyme in scale production and some industrial applications.

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