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Extracellular Methionine Amino peptidase (MAP) Production by *Streptomyces gedanensis* in Solid-State Fermentation

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ABSTRACT

A bioprocess was developed for extracellular MAP production from Streptomyces gedanensis *by solid-state fermentation. Response surface methodology of Box Behken Design was performed to evaluate the interaction effects of most significant variables {inoculum size, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ and tryptone) on MAP production after the single parameter optimization and it resulted a maximum MAP production of 55.26 IU/g PUF after 120 h of fermentation. The concentrated crude MAP displayed a pH and temperature optimum of 8.5 and 50°C. By analyzing the thermal stability, the MAP was found to be stable in a temperature range of 50 to 55°C but lost about 50% of its activity at 65°C after 30 min. This is a first report of this kind of study for MAP.*

Key words: *Streptomyces gedanensis*, Methionine amino peptidase, Fermentation, Box Behnken design

INTRODUCTION

Methionine amino peptidase (EC 3.4.11.18; MAP) preferentially hydrolyzes the ubiquitous N-terminal methionine from proteins and peptide substrates that have a small, uncharged amino acid in the penultimate position (Bradshaw et al. 1998). Amino peptidases are important for the development of flavour in fermented milk products since they are capable of releasing single amino acid residues from oligopeptides. In the food industry, amino peptidases from *Aspergillus oryzae and A. sojae* play an important role in digesting peptides, producing free amino acids from soybean protein. We reported earlier the amino peptidase mainly leucine aminopeptidase from *S. gedanensis* and also demonstrated its use as tool for protein hydrolysate preparations with improved functional properties (Rahulan et al. 2011, 2012). Bacterial MAPs are targets for antimicrobial therapy (Vaughan et al. 2002) whereas the structurally similar human MAP-2 is a target for anticancer agents (Logothetis et al. 2001). Therefore, discovery of new inhibitors may offer hope for the treatment of microbial infections as well as cancer. *Streptomyces* amino peptidases are of particular interest for biochemical and biomedical applications and are also valuable for the preparation of a debittered protein hydrolysate in the food industry (Arima et al. 2006) because they are stable and have a low molecular weight, simple kinetics, high enzyme activity and broad substrate specificity.

Solid-state fermentation (SSF) using inert supports allows direct biomass estimation, cleaner enzymatic extractions and more homogenous aeration (Zhu et al. 1994). Response surface methodology (RSM), which has been extensively

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applied in the optimization of medium composition, conditions of enzymatic hydrolysis, fermentation, and food manufacturing processes (Cui et al. 2006), is the collection of mathematical and statistical techniques for experimental design, model development, evaluation factors, and optimum conditions of different biotechnological bioprocess. The aim of the present study was to develop a bioprocess for MAP production using SSF by optimizing the process parameters using statistical tools and evaluating the basic characteristics of the concentrated crude MAP produced from *S. gedanensis.*

MATERIALS AND METHODS

Materials

L-methionine p-nitroanilide and 4-nitroaniline were obtained from Sigma, USA. The medium components were procured from Hi-media (Mumbai, India). All other analytical grade reagents were purchased from either Merck (Mumbai, India) or SD Fine Chemicals (Mumbai, India). UV 160A (Shimadzu, Kyoto, Japan) spectrophotometer was used for the enzyme assay. Cubical poly urethane foam (PUF), washed with distilled water and dried overnight at 70°C was used as inert support for SSF.

Microorganisms and Inoculum preparation

Streptomyces gedanensis IFO 13427 was a gift from Prof. Geroge Szakacs, Technical University, Budapest, Hungary. A loop full of the spores were transferred to the inoculum medium (50 mL in 250 mL Erlenmeyer flask), which contained (g/L); Beef extract, 1;Yeast extract, 1; Trypton, 2; Glucose, 10; $FeSO_4 \cdot 7H_2O$, 0.1 at pH 6.0. The flasks were incubated at 30°C and 200 rpm for 48 h and used as inoculums, which contained 1.2×10^9 CFU/mL.

Production media and Solid state fermentation (SSF)

Four different media were used to screen the production of methionine amino peptidase in SSF using poly urethane foam as inert support. The composition of the media was as follows (g/L); **Medium-1**: corn steep liquor (CSL), 10; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 1.0; NaCl, 2.0; Na_2CO_3, 0.5; Tween 80, 0.5; sucrose, 10; pH (before sterilization), 5.53. **Medium-2**: soybean powder, 10; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 1.0; $FeSO_4.7H_2O$, 0.1; NaCl, 2.0; beef extract, 5.0; yeast extract, 5.0; sucrose, 10; Tween 80, 0.3; pH (before sterilization), 6.53. **Medium-3**: sucrose, 10; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; NaCl, 2.5; $CaCl_2.2H_2O$, 0.1; beef extract, 5.0; Tween 80, 0.3; pH (before sterilization), 6.0. **Medium-4**: soybean powder, 10; sucrose, 10; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 1.0; NaCl, 2.0; Na_2CO_3, 0.5; Tween 80, 0.3; pH (before sterilization), 6.8. SSF was carried out in 250 mL Erlenmeyer flask containing 1.0 g PUF moistened with 10 mL nutrient medium autoclaved at 121°C for 20 min. Three millilitres (1.2×10^9CFU/mL) of 48 h old culture used as the inoculum, for each flask and incubated at 30°C and for six days. Samples were withdrawn as whole flasks in duplicates. Fermented solid matter from each flask was mixed with 100 mL distilled water, centrifuged at 9500 X g at 4°C for 15 min and the cell free supernatant was used for MAP assay. All the experiments were carried out in duplicates and the reported results are the average values with standard deviation.

Analytical methods

The methionine amino peptidase assay was adopted from Tan and Konings (1990) and used with slight modifications. The reaction mixture contained 1.0 mL of 2.5 mm of L-methionine p-nitroanilide (in 100 mm NaOH–Glycine buffer, pH 8.5), 1.0 mL 100 mM NaOH–Glycine buffer, pH 8.5 and 0.5 mL of the properly diluted supernatant. The well-mixed solution was incubated at 50°C for 15 min. The reaction was stopped by the addition of 1.0 mL of glacial acetic acid and the absorbance was measured at 405 nm. Assay was also carried out using appropriate substrate and enzyme blanks. One International Unit of enzyme activity is defined as the amount of enzyme that hydrolyses 1 µM of methionine p-nitroanilide per min. Standard curve was plotted using 4-nitroaniline. The protein concentration was measured by the Folin phenol reagent method (Lowry et al. 1951).

Experimental design and Data analysis

Different extraction systems (NaOH-glycine buffer, pH 8.5; Tris-HCl, pH 7.0; 0.1% of Tween 80 and distilled water) were used for MAP extraction from the fermented matter. In order to study the effect of pH on MAP production, initial pH of the moistening solution (medium) was set at 5.0 - 8.5 using 1N HCl or NaOH. The influence of initial moisture content on MAP production was studied by adjusting the moisture of PUF to

different levels by varying the volume of distilled water. Studies were also carried out to find out the efficacy of carbon source supplementation (starch, cellulose, dextrose, inulin and maltose) on MAP production. Different organic nitrogen sources (beef extract, yeast extract, tryptone, peptone and casein at 0.4%, w/w) were used to supplement the medium to check their influence on MAP production. After the identification of significant factors affecting the production of MAP in SSF by 'one variable at a time strategy', response surface methodology of Box- Behken design was employed to study the interaction effect of significant factors.

Box Behken design (BBD)

The significant variables such as inoculum size, $(NH_4)_2SO_4$ concentration, $MgSO_4.7H_2O$ and tryptone were chosen as the critical variables and designated as X_1, X_2, X_3 and X_4, respectively. The experimental design consisted of 29 runs and the independent variables were kept at three different levels. All the experiments were carried out in duplicate and the average of MAP production obtained was taken as the dependent variable or response (Y). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination, R squared (R^2) value. For each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated. Design Expert (Ver.6.0) by STATEASE Inc., Minneapolis, USA was used in this investigation.

Characterization of concentrated crude enzyme

The crude enzyme sample collected after the fermentation was concentrated by precipitation with 60-90% ammonium sulphate. The precipitate was dissolved in 50 mm NaOH-glycine buffer (pH 8.5) and dialyzed against the same solution. The active protein fraction obtained by ammonium sulfate precipitation was applied onto the Q Sepharose HP column XK50 (Amersham Biosciences Ltd., UK), previously equilibrated with 25 mm sodium phosphate buffer (pH 6.5). The proteins were eluted at 60 mL/h with a linear NaCl gradient (0.0–1.0 M) and collected in 1.0 mL fractions. The active fractions were pooled and subjected to pre-packed Sephacryl 100-HR column (Amersham Biosciences Ltd., UK) equilibrated with 50 mM NaOH-glycine buffer (pH 8.5). Proteins were eluted at 60 mL/h and collected in 1.0 mL fractions and the active fractions were pooled and subjected to biochemical characterization studies.

Effect of temperature and pH on MAP activity

The reaction was carried out at various temperatures ranging from 40 to 65°C and the enzyme activity at different temperature points were compared to find out the optimum temperature in each case. Similarly, the enzyme assay was carried out at different pH levels, such as 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 and 10.5. Three different buffers were used, 100 mm sodium phosphate buffer (pH 6.5–7.0), 100 mm Tris-HCl buffer (pH 7.5–8.0), and 100 mm NaOH-glycine buffer (pH 8.5–10.5).

Influence of metal ions on enzyme activity

To study the role of metal ions on enzyme activity, eight metal ion salts were applied in three different concentrations (2, 5 and 10 mm) in the reaction mixture. The following salts were used: $CaCl_2.2H_2O$; $CoCl_2.6H_2O$; $CuCl_2.7H_2O$; $MgCl_2.2H_2O$ and $ZnCl_2.2H_2O$, $FeCl_3$, $FeCl_2$ and $NiCl_2$. Enzyme assay was carried out at 50°C and pH 8.5 for 15 min.

Temperature stability

Thermal stability of the enzymes was determined following Karadzic et al. (2002).The concentrated enzyme sample was incubated at pH 8.5 at various temperatures (50, 60 and 65°C) for different time intervals (0, 5, 10, 15, 30, 45 and 60 min). After the heat treatment, samples were cooled and were subjected to enzyme assay at 50°C for 15 min. To study the role of metal ions on temperature stability, metal ion salts such as $CoCl_2.6H_2O$ was added in 2 mM concentrations to the reaction mixture and the stability was tested.

RESULTS AND DISCUSSIONS

Results of single parameter experimental approach to identify the significant factors for MAP production in SSF are shown in Table 1. The medium-1 had highest MAP activity (Table 1) at 120 h fermentation and was selected for further studies. Studies on the effect of incubation temperature and extraction system showed that the maximum MAP recovery was obtained by distilled

water at 30°C (Table 1). A significant increase in MAP activity (39.36± 0.00 IU/g PUF) was obtained when initial moisture content of the inert support was adjusted to 91.96%. Moisture content is a critical factor on SSF processes because this variable has influence on growth and biosynthesis and secretion of different metabolites (Chi et al. 2003). Supplementation of nitrogen and carbon sources also influenced the enhancement of enzyme production (Table 1).

The results of response surface methodology of Box Behken design experiments with different combinations of four variables are presented along with the mean predicted and observed responses in Table 2. The maximum MAP production was 57.38 IU/g PUF in run no 23 while the minimum MAP was 15.43 IU/g PUF in run no 12, which indicated that choosing appropriate cultivation conditions could evidently enhance the yield of MAP. The results suggested that the MAP production from *S. gedanensis* had a higher enzyme activity at 3.0 mL of inoculum size ($1.2X10^9$ CFU/mL), 0.275% (w/v) $MgSO_4.7H_2O$, 0.275% (w/v) $(NH_4)_2SO_4$ and 0.55 % (w/v) of tryptone.

The regression equation obtained after ANOVA indicated the determination coefficient (R^2) as 0.9733 for MAP production in SSF, which implied that the statistical model could explain 97.33 % variability in the response in reasonable agreement with the adjusted R^2 of 0.9465. Contour plots of the interaction between $MgSO_4.7H_2O$ concentration (B) and tryptone (C) showed that (Fig. 1A) a higher level of both factors led to negative impact on MAP production. It was noticed that initially MAP production increased gradually with increase in the value of $MgSO_4.7H_2O$. However, under optimal $MgSO_4.7H_2O$ concentration (B), excessive increase of tryptone (C) did not increase the yield of MAP activity. Fig. 1(B) represented the interaction between tryptone (C) and inoculum size (D) on MAP activity while other variables were kept at constant level. The results showed that MAP production increased gradually up to the middle level of both the factors and thereafter the enzyme yield declined.

Hence, by keeping an appropriate level of such key variables, the whole process could be converted to an economically feasible process. After the statistical optimization of significant variables, the yield increased to 57 IU/g PUF.

Table 1 - Optimized conditions of parameters for MAP production under SSF.

Optimized parameters	Optimized conditions	MAP activity (IU/gPUF) ±Standard deviation
Medium	Medium -1	28.99±0.05
Incubation temperature	30°C	30.97±0.02
Inoculum size	2; (1.2×10^9 CFU/mL)	30.92±0.05
Extraction system	Distilled water	31.27±0.01
Initial pH	5.50	31.51±0.01
Moisture content (%)	91.96 %.	39.36± 0.00
CSL concentration (control)	1% (w/v)	38.95± 0.02
Organic complexes	Tryptone; 0.4% (w/v)	41.96±0.00
Inorganic nitrogen	$(NH_4)_2SO_4$; 0.1% (w/v)	43.34±0.01
Carbon sources	Starch; 0.1% (w/v)	44.58±0.04

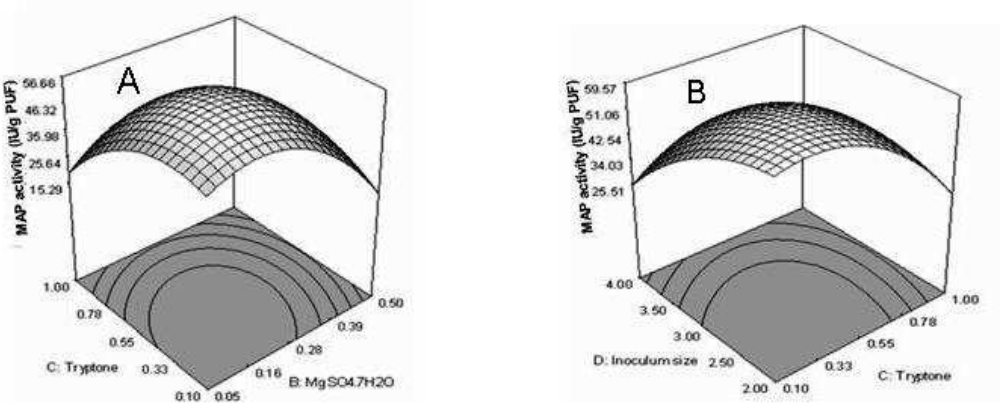

Figure 1 - A- shows the response surface graph of the interaction between $MgSO_4.7H_2O$ concentration (B) and tryptone (C) and **B-** shows the interaction effect of tryptone (C) and inoculum size (D) on MAP production in SSF by *S. gedanensis.*

Table 2 - Experimental design and results of response surface methodology of BBD for MAP production in SSF using inert support

Run	A $(NH_2)_4SO_4$ (% w/v)	B $MgSO_4.7H_2O$ (% w/v)	C Tryptone (% w/v)	D Inoculum size (mL)	MAP activity (IU/g PUF)	
					Actual value	Predicted value
1	0.275	0.275	0.55	3	52.14	53.92
2	0.050	0.050	0.55	3	46.35	45.35
3	0.275	0.275	0.55	3	53.28	53.92
4	0.050	0.500	0.55	3	15.48	15.87
5	0.050	0.275	1.00	3	25.46	21.92
6	0.500	0.275	0.55	2	44.45	42.18
7	0.275	0.275	0.55	3	54.63	53.92
8	0.275	0.275	0.10	2	56.26	57.68
9	0.275	0.500	0.10	3	18.46	17.02
10	0.050	0.275	0.55	2	52.26	56.61
11	0.275	0.275	0.55	3	52.16	53.92
12	0.275	0.500	1.00	3	15.43	15.29
13	0.275	0.050	0.55	4	29.38	27.14
14	0.500	0.275	1.00	3	36.28	35.82
15	0.275	0.275	1.00	2	26.24	27.14
16	0.500	0.275	0.10	3	39.24	38.57
17	0.275	0.275	0.10	4	23.48	25.51
18	0.050	0.275	0.10	3	52.26	48.50
19	0.050	0.275	0.55	4	20.36	23.91
20	0.275	0.050	1.00	3	16.56	19.28
21	0.275	0.275	1.00	4	26.21	26.73
22	0.500	0.500	0.55	3	26.49	30.42
23	0.275	0.275	0.55	3	57.38	53.92
24	0.275	0.050	0.55	2	52.56	49.12
25	0.275	0.500	0.55	4	16.69	15.91
26	0.275	0.050	0.10	3	45.46	46.87
27	0.500	0.050	0.55	3	32.24	34.78
28	0.500	0.275	0.55	4	45.38	42.31
29	0.275	0.500	0.55	2	28.48	26.58

Enzyme preparation

The crude MAP from *S. gedanensis* was concentrated by selective fractionation with ammonium sulfate and the two chromatographic steps resulted an enzyme preparation of 15.02 IU/mg (Table 3). The summary of enzyme preparation is shown in Table 3. The enzyme was purified near 600 -fold from the crude extract, with a yield of 6.31% (Table 3). MAP concentrated from *S. gedanensis* was characterized to explore its properties for industrial applications.

Table 3 - Summary of MAP preparation from *Streptomyces gedanensis.*

Concentrating steps	Total activity (IU/mL)	Total protein (mg/mL)	Specific activity (IU/mg)	Purification fold	Purification Yield (%)
Crude enzyme	171.31	62.00	0.37	1.00	100.00
$(NH_4)_2SO_4$ precipitation	69.00	15.70	1.93	5.21	40.20
Ion exchange (Q-Sepharose)	67.26	5.67	11.86	32.05	39.24
Gel-filtration(sephacryl-100 HR)	10.82	0.72	15.02	641.6	6.316

Enzyme characterization

Effect of temperature and pH on MAP activity

The enzyme was most active at 50°C and more than 60% of relative activity was found from 45 to 65°C (data not shown). Amidolytic activity against Met-p-NA was optimal at pH 8.5 (data not shown) but the activity rapidly declined after pH 9.5.

Effects of metal ions on enzyme activity

The effect of different metal ions on MAP activity was studied. Evidently Co^{2+} at 2 mm concentration enhanced the activity of MAP and was strongly inactivated by Ni^{+}, Fe^{2+}, Cu^{2+} and Zn^{2+}(data not shown). This collectively suggested that MAP was a Co^{2-}dependent metallo peptidase like Met AP of

Bacillus stearothermophilus (Chung et al. 2002). Different metal ions are known to activate various MAPs *in vitro* - Co2+ and Zn2+ for *E. coli* MetAP (Lowther et al. 1999); Co2+ and Mn2+ for human MetAP (Li and Chang 1996).

Thermal stability and metal ion influence on thermo-stability of MAP

Figure 2A shows the results of thermal stability of the MAP preparation. Incubation for 1 h at 50 and 55°C at pH 8.5 resulted in 25% relative activity loss. The activity was retained around 53 and 25 % of the initial activities even after 45 min of incubation at 60 and 65°C, respectively, but 99% of activity was lost at 65°C at 1 h incubation (Fig. 2A).Co^{2+} ions at 2 mm concentration were selected to examine their effect on heat stability of purified MAP from *S. gedanensis* (Fig. 2B). Co^{2+} improved the thermal stability of MAP. Heat treatment with Co^{2+} at 50 and 55°C did not influence the enzyme activity; thus, the enzyme was thermo-stable at this temperature. However, during the incubation at 60 and 65°C, the enzyme preparation retained around 65 and 59% relative activities even after 60 min incubation compared with that of the activity in the absence of Co^{2+}ions (Fig. 2B).The thermal stability of a protein is determined by many factors, which include hydrogen bonding networks, hydrophobic interactions, optimized core packing, salt bridges, and the reduction of the entropy of unfolding (Vieille and Zeikus 1996).

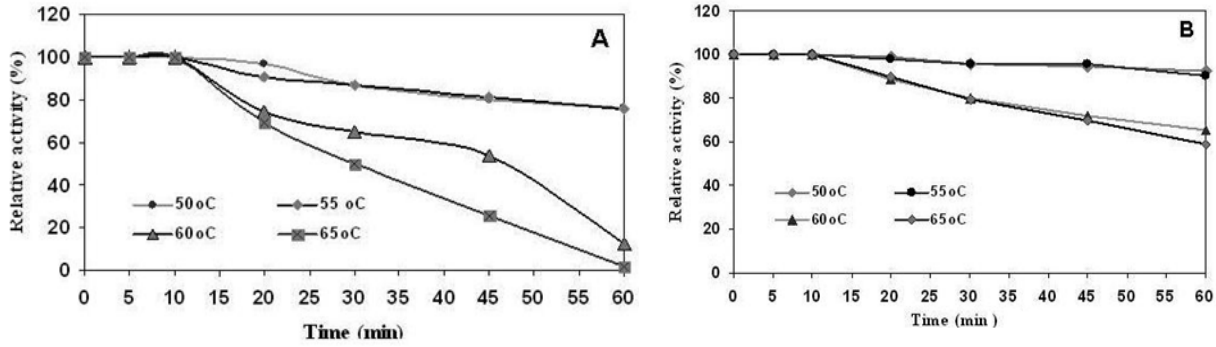

Figure 2 - A- shows the temperature stability of crude MAP and B- shows the Co2+ metal ion influence on MAP stability at various temperatures.

CONCLUSIONS

To the best of our knowledge, there is no report on extracellular MAP production from *S. gedanensis* in SSF. The present study revealed the feasibility of developing a bioprocess for MAP production and the results were encouraging for a possible scale-up of MAP production.

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