Extracellular proline aminopeptidase production by Streptomyces lavendulae ATCC14162 under solid-state fermentation

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This study presents extracellular Proline aminopeptidase (PAP) production by Streptomyces lavendulae ATCC 14162 under solid state fermentation (SSF). After optimization of few significant parameters (particle size of selected substrate, inoculums size, initial moisture level and incubation time), maximum PAP production was found to be 83.07 IU/gds. This is the first report on extracellular PAP production using SSF by a Streptomyces culture.

Keywords: Proline aminopeptidase (PAP), Proline-pNA, Streptomyces lavendulae, Solid-state Fermentation (SSF)

Introduction
Microbial aminopeptidases play important roles in utilization of exogenous proteins as a source of essential amino acids that can be utilized for protein synthesis, generation of metabolic energy and recycling of reduced cofactors. Proline aminopeptidase (PAP) specifically releases amino-terminal proline from peptides, and recognized for their debittering activity and contribute to flavor of final dairy product. Aminopeptidases and their microbial producer strains have important role in manufacture of protein hydrolysates and protein rich fermented products derived from soy, meat, milk, cereals etc. Pharmaceutical applications are being directed to control their activity in pathophysiological processes and development of diagnosis tools and markers of physiological pathways. Streptomyces aminopeptidases are of particular interest for biochemical, biomedical applications and also valuable for the preparation of a debittered protein hydrolysate in the food industry. Production of substrate specific aminopeptidases is reported by Lactobacilli, Streptomyces sp. etc; however, mostly they are intracellular in nature. Hence to develop a commercially feasible bioprocess, extracellular enzymes have to be produced in a most effective manner. Solid-State fermentation (SSF) utilizes various agro-industrial wastes. Industrial fermentation greatly depends on composition of medium, and it has a profound effect on the physiology of microorganisms and is often associated with the product formation. After initial screening of Streptomyces cultures, S. lavendulae was selected to check for PAP activity in submerged fermentation. However, yield obtained was not good for making an economically feasible bioprocess.

This study presents growth and enzyme production in SSF using wheat bran as substrate for producing extracellular PAP in SSF.

Experimental Section
Microorganism, Substrate and Inoculum
S. lavendulae ATCC 14162 was procured from Technical University, Budapest and maintained on ATCC-5 agar medium (beef extract, 1; yeast extract, 1; tryptone, 2; glucose, 10; FeSO₄·7H₂O, 0.1 and agar, 20 g/l) at pH 6.5 at 30°C by monthly sub culturing and stored at 4°C. Wheat bran, rice bran and soybean were obtained from local market, sun dried and sieved using different filters to get particle size of different sizes. Polyurethane foam was cut into cubes and washed several times with warm water and dried at 4°C. Chromogenic substrate L-Pro-pNA was purchased from Sigma. From a fully sporulated petri plate, a loop full of spores was transferred to 50 ml ATCC-5 growth medium in 250 ml Erlenmeyer flask, which was incubated at 30°C and 200 rpm for 48 h. Homogenous culture, which contained 9 x10⁵ CFU/ml, was used as inoculum.

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Solid-state Fermentation (SSF) and Enzyme Extraction

Dry wheat bran (5 g) was taken in Erlenmeyer flasks (250 ml) and substrate was moistened with required amount of distilled water to get a desired initial moisture level prior to sterilization. After autoclaving, 5 ml of 48 h old inoculum (9 x10^4 CFU/ml) was added aseptically to each flask and thoroughly mixed with a sterile spatula. Inoculated flasks were incubated for 96 h, fermented matter was mixed with distilled water (1:10 w/v), stirred slowly at 30°C for 1 h followed by filtration through muslin cloth. Filtrate was then centrifuged at 8000xg at 4°C for 10 min. Clear supernatant was used as crude enzyme. Dry weight was determined by drying fermented matter (1 g) in a hot air oven at 60°C for 16 h.

Optimization of Process Parameters

Experiments were conducted to optimize particle size of substrate, initial moisture of SSF medium (60, 65, 70, 75 and 80%), inoculum size [2 to 5 ml (9x10^4 CFU/ml)], incubation time and effect of supplementation of additional sources of carbon [glucose, lactose, starch, cellulose and glycerol (0.1% w/w)] and nitrogen [casein, yeast extract, beef extract, tryptone and peptone (0.1% w/w)]. Substrates used for screening include wheat bran, rice bran, soybean powder and inert Poly urethane Foam (PUF). When all the natural substrates were moistened with distilled water, inert PUF was impregnated with ATCC 5 nutrient medium. A time course study was conducted to check the effect of incubation period on enzyme production and was investigated over a period of 5 days at an interval of 24 h. Different parameter optimization experiments were carried out by keeping previously optimized conditions constant.

Enzyme Assay

Reaction mixture [L-Pro-pNA, 2.5 mmol l^-1; Tris HCl buffer (pH 8.5), 50 mM; and enzyme sample, 50 μl] for PAP was assayed at 37°C for 10 min as reported on 96-well plates using a microplate reader (Model 680XR, Bio-Rad, USA). Enzyme activity (1 IU) is defined as the amount of enzyme that hydrolyses 1 μM of L-Pro p-nitroanilide per min of reaction and calculated per gram dry substrate.

Electron Microscopy

Growth of S. lavendulae on wheat bran particles was characterized using an electron microscope at a magnification of 1,500 x for both native and fermented substrates using JEOL JSM-5600 Scanning Electron microscope. Fermented sample (96 h) was adequately dried and mounted on a brass stud followed by a mild gold coating and was subjected to electron microscopy.

Results and Discussion

Morphology and Growth on Solid Substrate

Morphology of S. lavendulae was observed under microscope after Gram staining. Strain appeared as gram positive with filamentous and irregular hyphae. Hyphal anchoring of culture on the surface of wheat bran during SSF was shown by SEM images (Fig. 1).

Evaluation of Agro-industrial Residues as Substrates for SSF

Among various substrates used for growth and enzyme production, only wheat bran supported adequate
growth and production (80.84 IU/gds) of PAP (complete data not shown). With inert synthetic substrate, growth was poor and thus the yield, indicating presence of other nutrient factors in wheat bran that favored enzyme production. Wheat bran has advantage of low cost and all time availability. In SSF, wheat bran has been a highly reported substrate for producing many of the microbial enzymes, bioactives and antibiotics etc.14. Widespread suitability of wheat bran may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions, thus providing a large surface area15.

**Effect of Particle size on PAP production**

Coarse (C) (3-5 mm) and fine particles (F) (<1 mm) of wheat bran were used in different combinations (1C; 1F:1C; 1F:3C; 1F:2C; 1F; 1F:1C; G- 1C); b) initial moisture; c) inoculum size ((9x10^4 CFU/ml); d) incubation time; and e) carbon sources

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Fig. 2 δ Effect on PAP production (control - wheat bran without any supplementation) using Streptomyces lavendulae of: a) particle size [A- 1F (Fine); B- 3F:1C (coarse); C- 2F:1C; D-1F:2C; E- 1F:3C; F- 1F:1C; G- 1C]; b) initial moisture; c) inoculum size ((9x10^4 CFU/ml); d) incubation time; and e) carbon sources
exchange leading to nutrient and bacterial cell leaching. Extremely small particles reduce mass transfer considerably\textsuperscript{16}.

**Effect of Initial Moisture on PAP Production**

Initial moisture controls enzyme production and a maximum of 80.94 IU/gds was obtained at 75% moisture (Fig. 2b). Required initial moisture (75%) is contributed by both distilled water and water content in inoculum. At lower moisture, growth was meager (data not included) and hence enzyme production. When an inert substrate like PUF was used, a relatively high moisture level (90%) was observed and enhanced yield of leucine aminopeptidases using *S. gedenensis*\textsuperscript{17}. Wheat bran may have a better capacity to hold the moisture for longer time than inert materials and thus 70-80% moisture found to be sufficient. Higher and lower hydration results less enzyme activity, may be because low moisture content is unfavourable for microorganism\textsuperscript{18}’ growth and at higher moisture content, void space within solid phase is filled with water and air is driven out, affecting growth of organisms and also enzyme production.\textsuperscript{19} Moisture content and water activity was always a crucial factor in any SSF process because this variable had influenced growth and biosynthesis of different metabolites including enzymes\textsuperscript{9}.

**Effect of Inoculum Size on PAP Production**

SSF was carried out with varying amount of inoculum with initial moisture of 75% and incubated at 30°C. Enzyme yield was maximum (78.75 IU/gds) at inoculum size of 5 ml (9x10\textsuperscript{4} CFU/ml) (Fig. 2c). Inoculum size determines biomass production on solid medium\textsuperscript{14}. An increase in inoculum size would ensure a rapid proliferation and biomass synthesis. However, when competition for nutrients become evident, there would be a decrease in metabolic activity of organism. At optimum inoculum size for enzyme production, there is a balance between proliferating biomass and availability of nutrients\textsuperscript{16}.

**Effect of Incubation Period on PAP production**

Using optimum moisture and inoculum size, maximum enzyme titre was noticed at 96 h of incubation (Fig. 2d) and is in linear to the growth (data not shown). In most of the organisms, enzyme production gradually increases during exponential phase and a maximum is attained towards the end of this phase or during stationary phase\textsuperscript{19}.

**Effect of Carbon and Nitrogen Source Supplementation on PAP Production**

Wheat bran based SSF medium supplemented with additional carbon sources revealed that supplementation of glucose polymers (starch and cellulose) doesn’t had any deteriorating effect on enzyme yield and in fact they slightly favored enzyme production (Fig. 2e). However, availability of free monosaccharides (lactose and glucose) resulted in a lower enzyme titre, could be due to carbon sources may be used for enhanced growth and within 96 h production may not have reached to the maximum titre and thus a longer incubation may be needed to see the maximum titre or there could be a kind of feedback inhibition. When polymer sugars are used there could be a controlled release of monomer glucose and that could be consumed by the organism and when released, hence no feed back inhibition due to glucose.

Supplementation with nitrogen sources showed no enhancement in enzyme production compared to control with wheat bran alone (complete data not shown). In fact, yield was reduced in many cases to the range of 50-60 IU/gds from control level of 75-80 IU/gds. This study revealed that wheat bran may be sufficient to support growth and enzyme production and is very vital while considering economical scale up of bioprocess without any sort of further supplementation.

**Conclusions**

*S. lavendulae* was successfully cultivated under SSF using wheat bran as support as well as a nutrient medium. During present SSF process, a significant production of an extracellular PAP enzyme was noted and maximum enzyme yield was 83.07 IU/gds. However, it was 1.5-2 IU/ml when liquid fermentation was adopted\textsuperscript{12}. This study opened a new avenue for an economically viable bioprocess for producing PAP enzyme through SSF. Ability of *S. lavendulae* to produce such a high titre of extracellular aminopeptidase can be explored in future to utilize it for food industry to make protein hydrolysates preparations with less bitterness or for peptide synthesis in pharmaceutical industry.

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