Turk J Biol
36 (2012) 665-671

doi:10.3906/biy-1201-26

Dipeptidyl peptidase IV production by solid state fermentation using alternative fungal sources

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Received: 23.01.2012 ● Accepted: 13.06.2012

Abstract: The present work was carried out for the production of dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) using *Aspergillus, Penicillium,* and *Rhizopus* strains under solid state fermentation conditions. Response surface methodology was applied for the optimization of the selected operational variables (corn flour, initial moisture content, and cultivation time) for DPP IV activity as the response. The optimal parameters of DPP IV activity for the independent variables, namely the amount of corn flour (% w/w), initial moisture content (% w/w), and cultivation time (days), were evaluated to be 2.44%, 60.85%, and 4.69 days, respectively, using *Aspergillus awamori* T116. The response for these results was also shown to be in very close agreement with the experimental data.

Key words: Dipeptidyl peptidase IV, *Aspergillus*, *Penicillium, Rhizopus*, solid state fermentation

Introduction

Studies on the dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) (CD26) enzyme have focused especially on human health. Besides being a useful marker for early diagnosis of diabetic nephropathy and tumor burden, DPP IV has roles in many biological processes including pancreatic cancer and rheumatoid arthritis (1-9). In addition to mammalian cells, DPP IV was isolated from insect and microbial cells (10). Mentlein mentioned the interaction of the DPP IV (CD26) enzyme with mammalian regulatory peptides in detail, and also mentioned that DPP IV had an important role not only in the regulation of the endocrine system, immune system, and gastrointestinal system, but also in the regulation of the nervous system (11). In humans, DPP IV was found intensively in kidneys and small intestine microvilli (12). Insufficient production of the DPP IV enzyme on the inner surface of the small intestine results in opioid effects of casomorphin and gluteomorphin structures, especially in autistics (13-18). DPP IV is a proline-specific enzyme (19). It hydrolyzes specifically the X-proline bonds found in casomorphin and gluteomorphin structures to overcome the opioid effect (20-22). In autistic individuals, some other important enzymes of digestion were also found to be produced insufficiently; however, the effects of the lack of those enzymes were not as dramatic as the effects of insufficient DPP IV (23,24). Although a casein-gluten free diet was recommended for autistic patients, the effectiveness of this diet is still under discussion (25-27). Another approach for the elimination of the opioid effect in autistics is an enzyme therapy that involves the use of DPP IV with other digestion enzyme formulations in capsules prior to, during, or after meals as dietary supplements (20,22).

The production of the DPP IV enzyme from microbial sources has mostly been from fungal and bacterial origins. Membrane-bound DPP IV was produced by *Lactococcus* strains (28,29). Extracellular DPP IV was isolated and characterized from *Aspergillus oryzae* by Tachi et al. (30). Doumas et al. (31) determined the DPP IV enzyme activity in the culture medium of *Aspergillus oryzae* 44 and TK3 and *Aspergillus nidulans*. Jalving et al. (32) cloned the dapB gene from *Aspergillus niger* and its nucleotide sequence as well as its protein sequence, which indicated that the gene encodes DPP IV. Other studies also reported DPP IV and DPP V gene cloning and characterization from *Aspergillus fumigatus* (33). A search of the databases results in *Aspergillus clavatus* NRRL 1 (ACLA_035780) (ACLA_048680), *Aspergillus flavus* NRRL 3357 (AFLA_110160) (AFLA_087160), *Aspergillus fumigatus* Af293 (NC_007197.1) (NC_007196.1), *Aspergillus terreus* NIH 2624 (NT_165930.1) (NT_165925.1), *Penicillium marneffei* ATCC18224 (PMAA_013400), and *Penicillium chrysogenum* Wisconsin 54-1255 (Pc20g06070) for DPP IV gene location and mRNA information. Production of the DPP IV enzyme from fungal sources by using submerged fermentation as well as solid state fermentation (SSF) technologies is possible. SSF offers well-known advantages over submerged fermentation, such as higher product titers, lower waste water output, reduced energy requirements, and simpler fermentation media. Hence, since it reflects the natural living conditions for fungal growth, SSF is mostly used for the cultivation of fungi.

The present work was focused on fungal sources and directed toward the production of DPP IV by SSF where economically feasible and renewable sources were used as substrates. Different fungal strains were screened based on their potential to produce DPP IV. Cultural and nutritional parameters such as initial moisture content, substrate concentration, and cultivation time for the DPP IV production were determined by the conventional "one variable at a time" approach, and the levels of the critical parameters were further optimized by response surface methodology (RSM) for dietary supplement.

Materials and methods

Materials

Aspergillus awamori, Aspergillus aculeatus, Aspergillus carbonarius, Aspergillus ficuum, Aspergillus foetidus, Aspergillus tubingensis, Penicillium canescens, Penicillium carneolutescens, Penicillium italicum, Penicillium varians, Penicillium rubrum, and *Penicillium solitum* were provided from the Department of Bioengineering Culture Collection (Ege University, Turkey), and *Rhizopus oryzae* NRRL 395 and *Rhizopus oryzae* NRRL 2286 were used in screening for their potential for DPP IV production. All cultures were maintained on potato dextrose agar slants at 4 °C.

Wheat bran was supplied from Altınbaşak Un Anonim Şirketi (İzmir, Turkey), and corn flour and wheat flour were supplied from Kitle Pazarlama Anonim Şirketi (İzmir, Turkey). Gly-Pro p-nitroanilide (G-0513; Sigma Chemical Co., USA) was used as a substrate in DPP IV assay. All chemicals were of analytical grade.

Inoculum and culture conditions

All of the tested *Aspergillus*, *Penicillium*, and *Rhizopus* strains were grown on malt extract agar at 28 °C for 7 days. The spores were suspended in 0.2% (v/v) Tween 80 solution. One milliliter of spore suspension (10^7 conidiospores/mL, counted with a Thoma cell counting chamber) was used to inoculate 250-mL flasks containing 5.0 g of screening medium, including wheat bran and 0.7% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.1% $MgSO_4$, 2.0% corn flour, and 4.0% glucose (by weight of wheat bran on dry basis), adjusting the initial moisture of the medium to 55% (w/w) with distilled water. Medium was sterilized at 121 °C for 20 min. Incubation was carried out at 28 °C in an incubator.

After screening for the production of DPP IV, the selected fungi were grown at 28 °C in 250-mL flasks containing 5.0 g of wheat bran medium containing 2.0% corn flour or 2.0% wheat flour (by weight of wheat bran used on dry basis) as a substrate, and Vogel salt solution (Na-citrate.$2H_2O$, 2.5 g/L; KH_2PO_4, 5.0 g/L; $MgSO_4.7H_2O$, 0.2 g/L; NH_4NO_3, 2.0 g/L; $CaCl_2.2H_2O$, 0.1 g/L) was used as the moisturizing liquid instead of distilled water. After sterilization at 121 °C for 20 min, the initial moisture of the medium was 55

% (w/w). The levels of the critical parameters were further optimized with the same medium mentioned above.

Determination of initial moisture content

Initial moisture content was measured by drying a preweighed amount of substrate in Mettler Toledo halogen moisture analyzer equipment until it reached a constant weight. Weight loss in the original sample was recorded as mass of evaporated water (g) and converted to percent moisture content (w/w).

DPP IV assay

DPP IV was extracted by adding 25 mL of 0.2% (v/v) Tween 80 solution to each flask containing the fermented medium at 25 °C. Samples were mixed with a glass rod for 10-min intervals and 30 min of contact time was found to be sufficient for the extraction process. After extraction, the mixture was filtered through muslin cloth and centrifuged (10,000 rpm, 10 min, 4 °C). The supernatants were assayed for DPP IV activity.

DPP IV assay was performed according to Sigma's quality control procedure (34). The reaction mixture was prepared with 100 µL of supernatants and 100 µL of 1 mM Gly-Pro p-nitroanilide as a substrate solution in 96-well plates. It was incubated for 15 min at 37 °C. DPP IV activity was assayed at 405 nm by measuring the degree of substrate hydrolysis and comparing it with the p-nitroaniline (N-2128, Sigma Chemical Co.) concentration curve. A unit of enzyme activity was defined as the amount of the enzyme (Unit) producing 1 µmol p-nitroaniline/min. Results are expressed as mU/mL crude extract.

Experimental design

Design Expert (version 7.1.6, Stat-Ease, Inc., USA) software was used for regression and graphical analysis of the data obtained. The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The statistical significance of the regression coefficients was determined by Student's t-test, the second order model equation was determined by Fisher's test, and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The optimum values of the variables were obtained by graphical and numerical analysis using the Design Expert program, based on the criterion of desirability.

Results and discussion

Aspergillus oryzae, *Aspergillus fumigates*, *Aspergillus niger*, and *Aspergillus nidulans* were reported to possess mRNA expression of DPP IV (30-33). In our study, not only *Aspergillus* strains but also *Penicillium* and *Rhizopus* strains were investigated for their DPP IV production performance, since they are mentioned in some patented productions (22,35). Figures 1 and 2 indicate the time course of DPP IV production by *Aspergillus* and *Penicillium* strains. The maximum DPP IV activities obtained from *Rhizopus oryzae* NRRL 395 and *Rhizopus oryzae* NRRL 2280 were 59 mU/mL and 48 mU/mL, respectively, for 2 days of cultivation.

All tested fungal strains indicated the ability to produce DPP IV. Among them, *Aspergillus awamori* T116 and *Penicillium solitum* CD120 were selected for further experiments since they presented the highest DPP IV activities.

Selected strains were further tested on wheat flour-supplemented and corn flour-supplemented medium with Vogel's salts. The results are given in Table 1.

It was observed that *Aspergillus awamori* T116 showed higher DPP IV activity in corn flour-supplemented medium. Therefore, it was used in the RSM experiments. Experimental set-up procedures were planned to obtain a quadratic model consisting of 2^3 trials plus 6 star points and 3 replicates at the center point. The coded values of the independent variables, namely corn flour in % (X_1), initial moisture content in % (X_2), and cultivation time in days (X_3), and the experimental results of DPP IV activity in mU/mL for each case are presented in Table 2.

The regression analysis showed that the model can be expressed by Eq. (1), where the variables are in the form of coded values. Here, the model represents DPP IV activity (Y) as a function of corn flour (X_1), initial moisture content (X_2), and cultivation time (X_3).

$$Y = 444.55 + 3.36X_1 + 21.13X_2 + 69.06X_3 + 30.50\,X_1\,X_2 - 29.60\,X_1\,X_3 + 1.01\,X_2\,X3 + 6.07\,X_1^2 - 31.41\,X_2^2 - 106.13\,X_3^2 \quad (1)$$

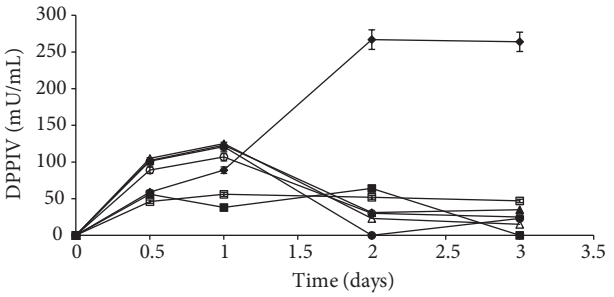

Figure 1. Production of DPP IV from *Aspergillus awamori* T116 (♦), *Aspergillus tubingensis* (●), *Aspergillus aculeatus* (○), *Aspergillus ficuum* (△), and *Aspergillus carbonarius* (□).

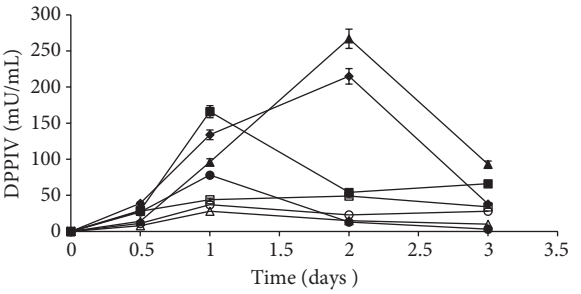

Figure 2. Production of DPP IV from *Penicillium solitum* CD120 (▲), *Penicillium canescens* T93 (♦), *Penicillium varians* T58 (□), *Penicillium italicum* R224 (●), *Penicillium carneolutescens* 252 (○), *Penicillium rubrum* R246 (Δ), and *Penicillium lanosum* R63 (■).

Table 1. Maximum DPP IV activities obtained in wheat flour-supplemented and corn flour-supplemented medium by *Aspergillus awamori* T116 and *Penicillium solitum* CD120.

Microbial source	**Medium**	**DPP IV activity** (mU/mL)
Aspergillus awamori T116	Wheat flour	349.2 (4 days)
	Corn flour	415.9 (4 days)
Penicillium solitum CD120	Wheat flour	42.0 (1 day)
	Corn flour	29.4 (1 day)

The model F-value was found to be 16.16, implying that the model was significant. The model F-value was calculated as the ratio of the mean square regression to the mean square residual. Thus, there was only a 0.07% chance that this "model F-value" occurs due to noise. Values of "Prob>F" being less than 0.0500 indicates that the model terms are significant. In this case, the model obtained was highly significant with 3 significant terms of X_3, X_2^2, and X_3^2, indicating that the linear or direct effect of cultivation time and quadratic effects of initial moisture content and cultivation time are a function of DPP IV activity. The determination coefficient (R^2) for the model was found to be 0.9541, indicating that only 4.59% of the total variations could not be explained by the model. The regression equation was represented by the 3D response surface graphics. The 3D response surfaces are presented in Figures 3-5, from which the DPP IV activity for different values of the variables can be predicted.

In the bioprocess yielding the maximum activity of DPP IV, initial moisture content and cultivation time are important variables because they both, individually and interactively, influence the response considerably. By solving the model equation, the optimal values of the test variables were found to be: U_1 (amount of corn flour) = 2.44% (w/w), U_2 (initial moisture content) = 60.85% (w/w), and U_3 (cultivation time) = 4.69 days with the corresponding Y = 471.1 mU/mL. DPP IV activity was determined as 462.6 mU/mL when optimized values were applied. This result is obviously in close agreement

Table 2. Experimental central composite design (CCD) runs in coded forms and corresponding responses.

Runs	Variables			Response
	X_1 corn flour (% w/w)	X_2 initial moisture content (% w/w)	X_3 cultivation time (days)	Y DPP IV activity (mU/mL)
1	−1	−1	−1	197.04
2	1	−1	−1	185.13
3	−1	1	−1	191.56
4	1	1	−1	342.86
5	−1	−1	1	399.09
6	1	−1	1	309.98
7	−1	1	1	438.88
8	1	1	1	430.54
9	−1.682	0	0	462.23
10	1.682	0	0	464.61
11	0	−1.682	0	364.54
12	0	1.682	0	350.25
13	0	0	−1.682	62.42
14	0	0	1.682	229.68
15	0	0	0	429.83
16	0	0	0	447.93
17	0	0	0	455.32

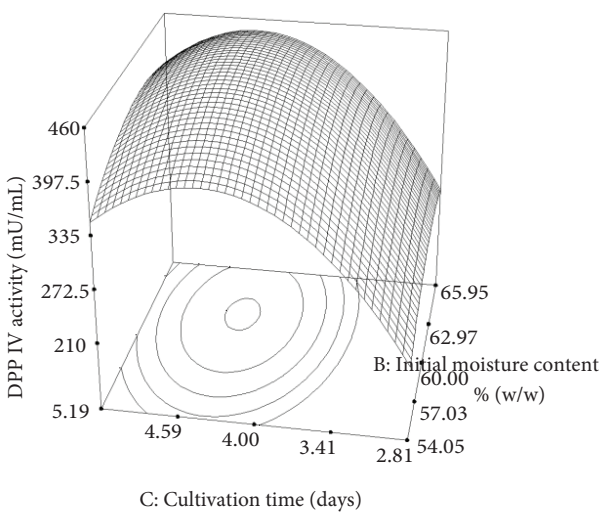

Figure 3. DPP IV activity as a function of initial moisture content and cultivation time.

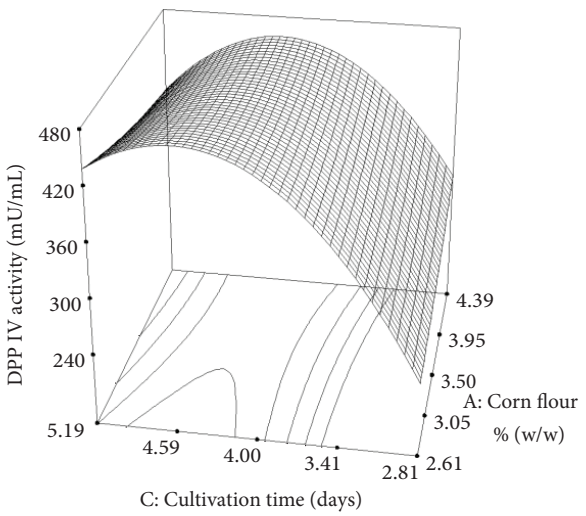

Figure 4. DPP IV activity as a function of corn flour percentage and cultivation time.

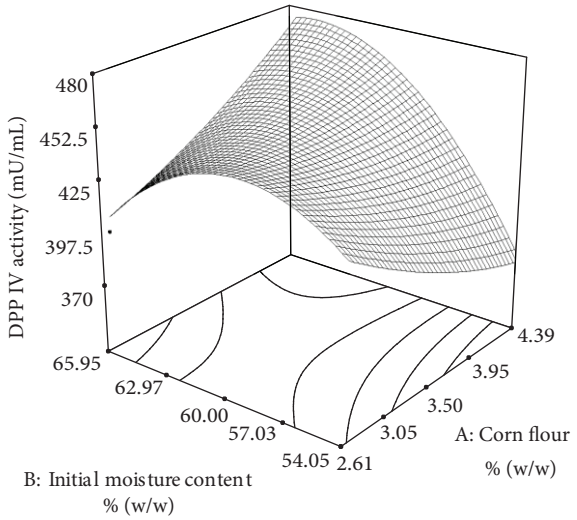

Figure 5. DPP IV activity as a function of corn flour percentage and initial moisture content.

with the model's prediction. RSM analyses show that the production of DPP IV does not depend strictly on corn flour. Thus, if there is an economic constraint about corn flour, a medium containing a lower amount of corn flour can be employed.

This study recommends a cost-effective way of producing the DPP IV enzyme from *Aspergillus awamori* T116. It is difficult to compare the amount of enzyme titers obtained in this study to those of other studies performed in this field, since different enzyme activity assays were applied in the literature. However, in a comparable study performed by Monod et al., it was mentioned that *Aspergillus* cells were chosen according to their capability of producing at least 100 mU/mL of DPP IV activity in submerged culture where wheat gluten was used in a minimal medium (35). Thus, according to these findings, it can be concluded that promising enzyme titers were obtained from this study.

Further studies will be conducted on purification of the enzyme and the finding of the optimal dosage in capsule form as a dietary supplement for autistics.

Acknowledgments

We thank Şevket Karaçancı (PhD) for using the Design Expert software (version 7.1.6, Stat-Ease, Inc.). This study was supported by TÜBİTAK, project number 105T195.

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