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Optimization of mycophenolic acid production in solid state fermentation using response surface methodology

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Mycophenolic acid (MPA) can be produced in solid state fermentation. An isolate of *Penicillium brevi-compactum* ATCC 16024 grown on moist wheat bran produced a titre of 425 mg per kg of wheat bran. Central composite rotatable design and response surface methodology were employed to derive a statistical model for media optimization towards production of mycophenolic acid. Five levels with a five factorial design were adopted. The correlation coefficient was 0.82, ensuring a satisfactory adjustment of the model to the experimental values. This statistical design was very effective in improving the titre of mycophenolic acid up to 3286 mg per kg of wheat bran.

Keywords: mycophenolic acid; solid state fermentation; media optimization; response surface methodology; *Penicillium brevi-compactum*

Mycophenolic acid (MPA) and its derivatives such as mycophenolate mofetil (MMF), have diverse biological properties such as antineoplastic [25], immunosuppressive [8], anti-inflammatory [1], antiviral [2], antipsoriasis [7] and antifungal activity [15]. Recent observations in experimental animal and human recipients of MMF showed this compound to have immunosuppressive properties [1]. Moreover, MMF in combination with cyclosporin-A (CyA) decreases the incidence of graft rejection to 17% in comparison with 60% in cases when only CyA was given to humans [23]. It is also reported that MMF has several advantages over CyA for maintenance therapy of organ graft recipients [8]. MMF is reported to be well tolerated with low toxicity [23].

MPA is produced by several species of *Penicillium* [3] in submerged culture. Production of enzymes through solid state fermentation (SSF) is well documented [19,21,22,24]. We were successful in producing lovastatin [20] and CyA [17] under SSF. In this communication, SSF is explored for production of MPA by *Penicillium brevi-compactum*. SSF involves the growth of microorganisms on moist solid substrates without free flowing water [4] and attracts a great deal of interest in view of its advantages and applications in industrial production of useful metabolites [13,14]. Filamentous fungi are often most suited to SSF for the production of several valuable metabolites since these conditions are similar to their natural habitat [11]. Experiments were conducted to optimize the media requirements using response surface methodology (RSM). This is a powerful tool in the optimization of a variety of applications such as media and process development [5,10,17,18,26,27], environmental factors on growth of microorganisms [12], and enzyme immobilization [6] etc. This technique generates contour plots from the linear, interaction and quadratic effects of two or more variables and fits the experimental data to calculate the optimal response of the system. MPA thus produced, was isolated and had properties compatible with the reference standard obtained from Sigma, St Louis, MO, USA.

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Materials and methods

Organism

A monospore isolate from *Penicillium brevi-compactum* ATCC 16024 was used. The culture is designated as DRCC 098 (Dr Reddy's Culture Collection). The culture was maintained on malt extract-yeast extract agar (MYA) slants.

Inoculum development

The organism was subcultured on a fresh MYA slant and incubated at 28 ± 1°C. After 10 days, the sporulated slants were suspended in 10 ml of saline containing 0.01% sterile Tween 80. Five millilitres of the spore suspension were inoculated into a 250-ml Erlenmeyer flask containing 50 ml of seed medium comprising (g L^{-1}): glucose 10; corn steep liquor 5; oat flour 10; tomato paste 40 and trace element solution (10 ml). The trace element solution consisted of: $FeSO_4 \cdot 5H_2O$, 1 g; $MnSO_4 \cdot 7H_2O$, 760 mg; boric acid, 56 mg; $CaCl_2 \cdot 2H_2O$, 100 mg; $CuSO_4 \cdot 5H_2O$, 25 mg; ammonium molybdate, 19 mg; $ZnSO_4 \cdot 5H_2O$, 200 mg and distilled water 1000 ml. The medium was adjusted to pH 5.5 before autoclaving it. The flasks were kept on a rotary shaker at 200 rpm for 48 h at 27 ± 1°C. Ten millilitres from this seed growth were added to 100 ml of second-stage seed medium taken in 500-ml Erlenmeyer flasks and grown on a rotary shaker at 200 rpm for 24 h at 27 ± 1°C. The second-stage seed medium was composed of (g L^{-1}): glucose 20; corn steep liquor 10; oat flour 20; tomato paste 80 and 1% trace element solution.

Solid state fermentation

Ten-gram samples of wheat bran were dispensed into 250-ml Erlenmeyer flasks and distilled water was added to get

an initial moisture content of 70% and the flasks were autoclaved for 1 h at 121°C. After cooling the flasks to room temperature, 5 ml of second-stage seed growth was added and the contents of the flask were thoroughly mixed. The flasks were incubated in an environmental chamber (Conviron Inc, Winnipeg, Manitoba, Canada) maintained at 25 ± 1°C and 80 ± 5% relative humidity (RH). Flasks were harvested each day from 1 to 8 days. The mouldy bran was extracted with 100 ml of ethyl acetate and the MPA titres were estimated by HPLC.

Evaluation and screening of nutrients

Eight carbon sources, two inorganic nitrogen sources, three organic nitrogen sources, six minerals and five complex organic nutrients were evaluated. The nutrients were added individually to the flasks in the concentrations as per Table 1. Eleven nutrients were then selected and further screened using a Plackett–Burman design [16]. The method was developed using Design Expert System (version 5.0.3, Stat-Ease Inc, Minneapolis, MN 55413, USA). The lowest and the highest concentration of each selected nutrient are given along with the design in Table 2. SSF medium consisted of 10-g samples of wheat bran with selected nutrients. The initial moisture content in each flask was adjusted to 70%. Flasks were sterilized for 1 h at 121°C. After cooling them to room temperature, flasks were inoculated with 5 ml of inoculum (30–35% PCV), and incubated at 25 ± 1°C and RH 85 ± 5% for 6 days. The effect of each nutrient on MPA production was determined by calculating the E-value as per the equation described elsewhere [9].

Table 1 Effect of nutrients on the production of mycophenolic acid by *P.brevi-compactum* in solid state fermentation

S. No.	Supplement used	Concentration (%)	Titre (mg kg^{-1} of bran)
1	Glucose	2.0	365
2	Sucrose	2.0	415
3	Maltose	2.0	658
4	Glycerol	2.0	426
5	Malto dextrin	2.0	387
6	Soluble starch	2.0	498
7	Lactose	2.0	438
8	Mannitol	2.0	746
9	Millet flour	2.0	667
10	Ragi flour	2.0	679
11	Jowar flour	2.0	469
12	Oat flour	2.0	441
13	Corn flour	2.0	663
14	$(NH_4)_2HPO_4$	1.0	669
15	NH_4NO_3	1.0	396
16	Corn steep liquor	1.0	330
17	Casein hydrolysate	1.0	619
18	Peptone	1.0	435
19	$ZnSO_4$	0.1	708
20	$FeCl_3$	0.1	469
21	$MgSO_4$	0.1	636
22	$CoCl_2$	0.1	398
23	$MnSO_4$	0.1	682
24	KCl	0.1	613

Table 2 Screening of potential nutrients and their ranking for mycophenolic acid production by Plackett–Burman design

S. No.	Nutrient	E-value	Ranking
1	Maltose	55	6
2	Mannitol	148	3
3	Millet flour	248	1
4	Ragi flour	152	2
5	Corn flour	–30	11
6	$(NH_4)_2HPO_4$	73	4
7	Casein hydrolysate	71	5
8	$ZnSO_4$	–2	10
9	$MgSO_4$	–1	9
10	$MnSO_4$	3	7
11	KCl	2	8

Optimization of selected nutrients using response surface methodology

A full factorial composite rotatable design (CCRD), developed using a Design Expert System (Version 5.0.3, Stat-Ease Inc, Minneapolis, MN 55413, USA), was used to study the five best nutrients selected from a Plackett–Burman design. A second order polynomial equation was used to study the interaction between dependent and independent variables

$$\eta_k = \Sigma\beta_{ko} + \sum_{I=1}^{5}\beta_{ki}\ x_i + \sum_{I=1}^{5}\beta_{kii}\ x_i^2 + \sum_{I=1,}^{4}\ \sum_{j=i+1}^{5}\beta_{kij}\ x_i x_j \quad (1)$$

where η_k is the dependent variable (predicted MPA titre in mg kg^{-1} of wheat bran) and β_{ko} is the regression coefficient at the centre; β_{ki}, β_{kii}, and β_{kij} are linear, quadratic, and second order coefficients, respectively. x_i and x_j are dependent coded variables for each factor. The coded values for the five levels used in this design are −2, −1, 0, 1 and 2 (Table 3). These variables are defined in the equation

$$x_i = \nu_i - \nu_{oi}/\lambda_i \quad (2)$$

where ν_i is the value of factor i at normal units, ν_{oi} is the factor i average value and λ_i is the step of each factor. The basal medium consisted of 10-g samples of wheat bran, supplemented with 0.1% each of $ZnSO_4 \cdot 5H_2O$,

Table 3 Experimental design to determine the optimum concentration of selected nutrients on MPA production

Factor (% w/w)	Coded symbol	Level				
		−2	−1	0	1	2
Mannitol	X_1	1	2	3	4	5
Ragi	X_2	5	10	15	20	25
Millet	X_3	5	10	15	20	25
$(NH_4)_2HPO_4$	X_4	1	2	3	4	5
Casein hydrolysate	X_5	1	2	3	4	5

$MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, and KCl. The basal medium was dispensed into a 250-ml Erlenmeyer flask and the concentration of nutrients to be optimized was added as per the design (Table 3). All other growth conditions were the same as described earlier. Fifty experiments were performed to determine the effect constants. The multiple regression analysis given in Equation 1, was used for each response to calculate the equation coefficients.

Analytical methods

MPA was estimated by high performance liquid chromatography (Shimadzu LC 10 A, Japan). The mobile phase consisted of potassium dihydrogen phosphate and acetonitrile in the ratio of 73 : 27. The pH was adjusted to 3.0 using phosphoric acid. The flow rate was maintained at 1.0 ml min^{-1}. A Novopak C18 (150 mm dia) column was used. The temperature of the column was maintained at 50°C with an operating pressure of 18 kg cm^{-2}. The HPLC profile was monitored at 305 nm.

NMR was recorded on a Varian, Gemini 200 spectrometer equipped with 5 mm ^1H and ^{13}C probe operating at 199.975 and 50.289 MHz, respectively. Samples were run in $CDCl_3$ with TMS as internal standard. FT-IR spectra were recorded in solid state as KBr dispersion using a Perkin-Elmer 1650 FTIR spectrophotometer. Standard MPA was procured from Sigma, USA.

Results and discussion

Kinetics of MPA production

Penicillium brevi-compactum DRCC 098 grown on moist wheat bran showed a steady increase in the production of MPA to a maximum titre of 425 ± 10 mg kg^{-1} of bran on the 6th day (Figure 1). The titre decreased to 350 mg kg^{-1} bran on the 8th day. Thus a fermentation time of 6 days was selected for all further experiments.

Evaluation and screening of nutrients

Based on experience gained in developing processes for secondary metabolites by SSF [17,20], a total of 24 nutrients were evaluated for their performance on production of MPA. When the nutrients were added separately to wheat bran, the titres varied between 330 mg and 746 mg of MPA kg^{-1} bran (Table 1). The nutrients which gave a considerable increase in titre over control were selected. Maltose and mannitol enhanced production of MPA to a greater extent than the other carbon sources studied. Amongst the nitrogen sources, diammonium hydrogen phosphate and casein hydrolysate improved the titres to 669 ± 25 and 619 ± 18 mg kg^{-1} of bran, respectively. Zinc sulphate, magnesium sulphate, manganese sulphate and potassium chloride showed higher titres amongst the minerals studied. Addition of millet (*Pennisetum typhoides* (Brum.f.) Stapf and Hubbard.) and ragi flour (*Eleusine coracana* Gaert.) to wheat bran improved the titres to 667 ± 16 and 679 ± 22 mg kg^{-1} of bran, respectively. Out of 24 nutrients studied, 11 showed a considerable increase in the titre of MPA compared to a control. To reduce the number of nutrients to be considered for media optimization, relative ranking of the nutrients was studied using a Plackett–Burman design.

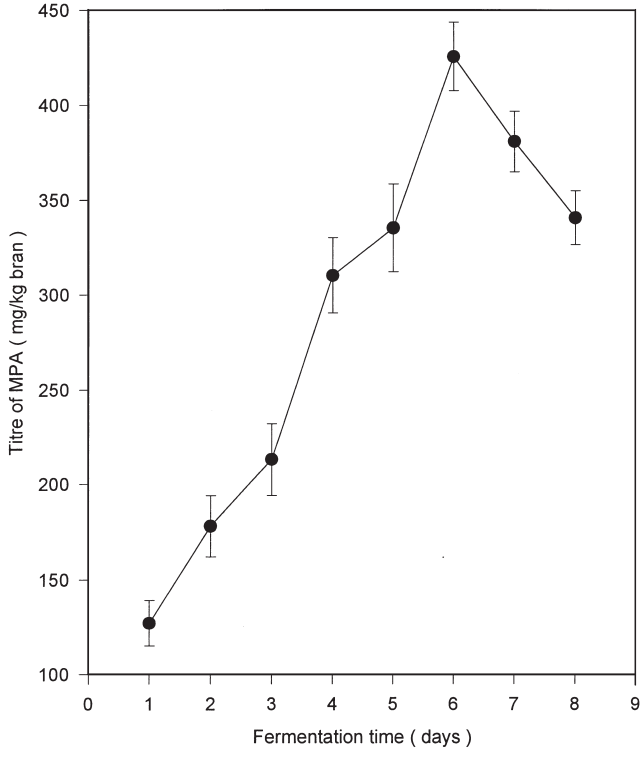

Figure 1 Kinetics of mycophenolic acid production by *Penicillium brevi-compactum* DRCC 098 in solid state fermentation.

Based on the E-values as shown in Table 2, mannitol, ragi flour, millet flour, diammonium hydrogen phosphate and casein hydrolysate were ranked as the best nutrients. Corn flour showed a negative E-value while minerals gave an E-value close to zero; this suggested that any further increase in the concentration of these nutrients, would not improve the titre of MPA.

Optimization of selected nutrients by response surface methodology

Based on the Plackett–Burman design, the best five nutrients specifically, mannitol, ragi flour, millet flour, $(NH_4)_2HPO_4$ and casein hydrolysate were selected for

Table 4 Second order polynomial coefficients

Coefficient	Response	Coefficient	Response
β_{k0}	2348.92	β_{k12}	23.56
β_{k1}	10.99	β_{k13}	37.44
β_{k2}	291.65	β_{k14}	–19.81
β_{k3}	–238.85	β_{k15}	–29.81
β_{k4}	405.49	β_{k23}	34.81
β_{k5}	–283.76	β_{k24}	100.31
β_{k11}	–143.41	β_{k25}	–28.81
β_{k22}	–37.78	β_{k34}	96.19
β_{k33}	–64.92	β_{k35}	–45.19
β_{k44}	–156.22	β_{k45}	–106.56
β_{k55}	–77.56		
	r^2	0.8268	

These second-order polynomial coefficients for experiments refer to Table 3.

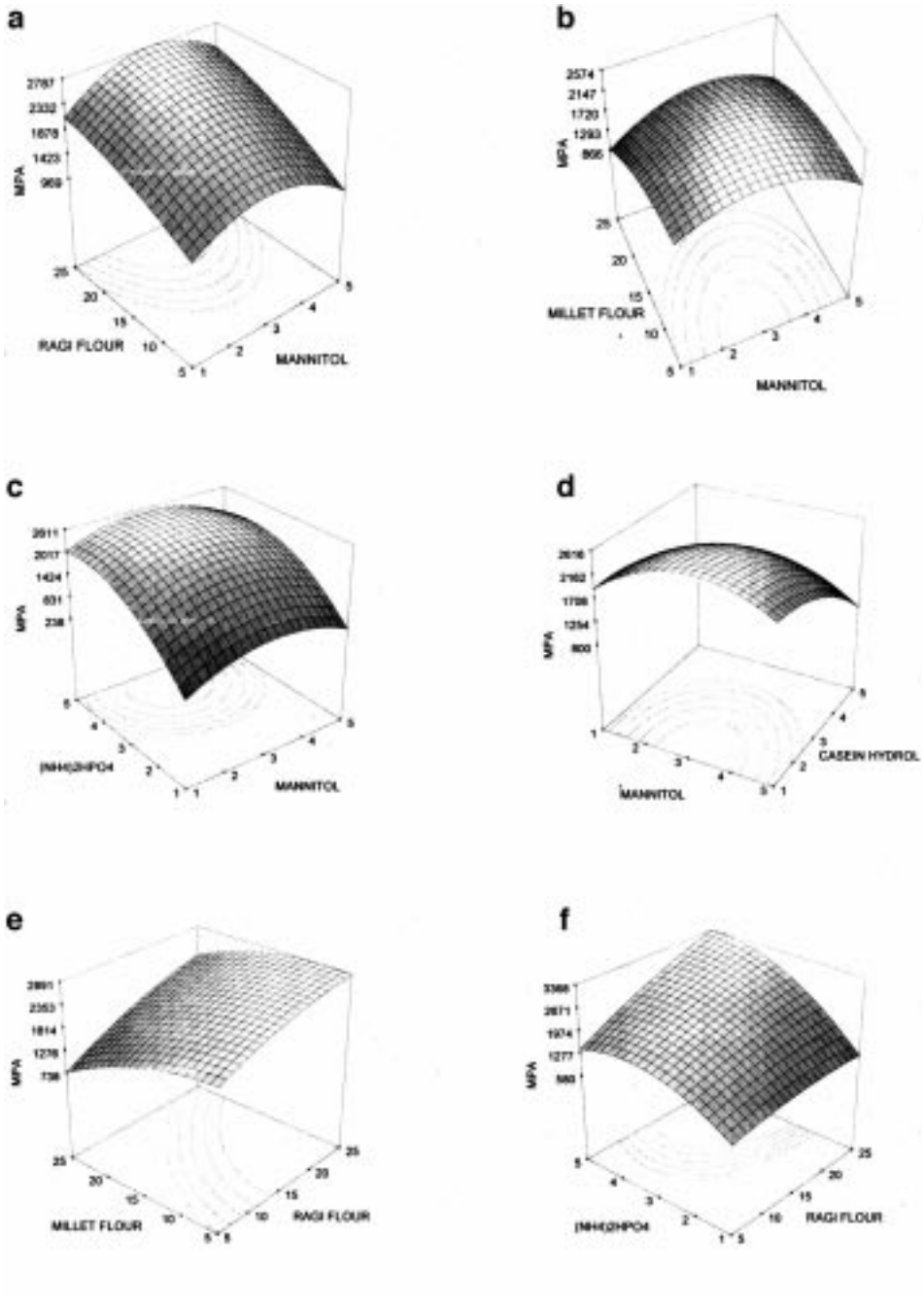

Figure 2 Interaction of nutrients on the production of mycophenolic acid by *Penicillium brevi-compactum* DRCC 098 in solid state fermentation. (a) Interaction of ragi flour and mannitol when millet flour, casein hydrolysate and $(NH_4)_2PO_4$ are kept at 15%, 3% and 3%, respectively. (b) Interaction of millet flour and mannitol when ragi flour, casein hydrolysate and $(NH_4)_2PO_4$ are kept at 15%, 3% and 3%, respectively. (c) Interaction of $(NH_4)_2PO_4$ and mannitol when millet flour, ragi flour, and casein hydrolysate are kept at 15%, 15% and 3%, respectively. (d) Interaction of mannitol, and casein hydrolysate when millet flour, ragi flour and $(NH_4)_2PO_4$ are kept at 15%, 15% and 3%, respectively. (e) Interaction of millet flour and ragi flour when mannitol, casein hydrolysate and $(NH_4)_2PO_4$ are kept at 3% each. (f) Interaction of $(NH_4)_2PO_4$ and ragi flour when millet flour, mannitol and casein hydrolysate are kept at 15%, 3% and 3%, respectively. (g) Interaction of casein hydrolysate and ragi flour when millet flour, mannitol and $(NH_4)_2PO_4$ are kept at 15%, 3% and 3%, respectively. (h) Interaction of $(NH_4)_2PO_4$ and millet flour when ragi flour, mannitol and casein hydrolysate are kept at 15%, 3% and 3%, respectively. (i) Interaction of casein hydrolysate and millet flour when ragi flour, mannitol and $(NH_4)_2PO_4$ are kept at 15%, 3% and 3%, respectively. (j) Interaction of casein hydrolysate and $(NH_4)_2PO_4$ when ragi flour, millet flour and mannitol are kept at 15%, 15% and 3%, respectively.

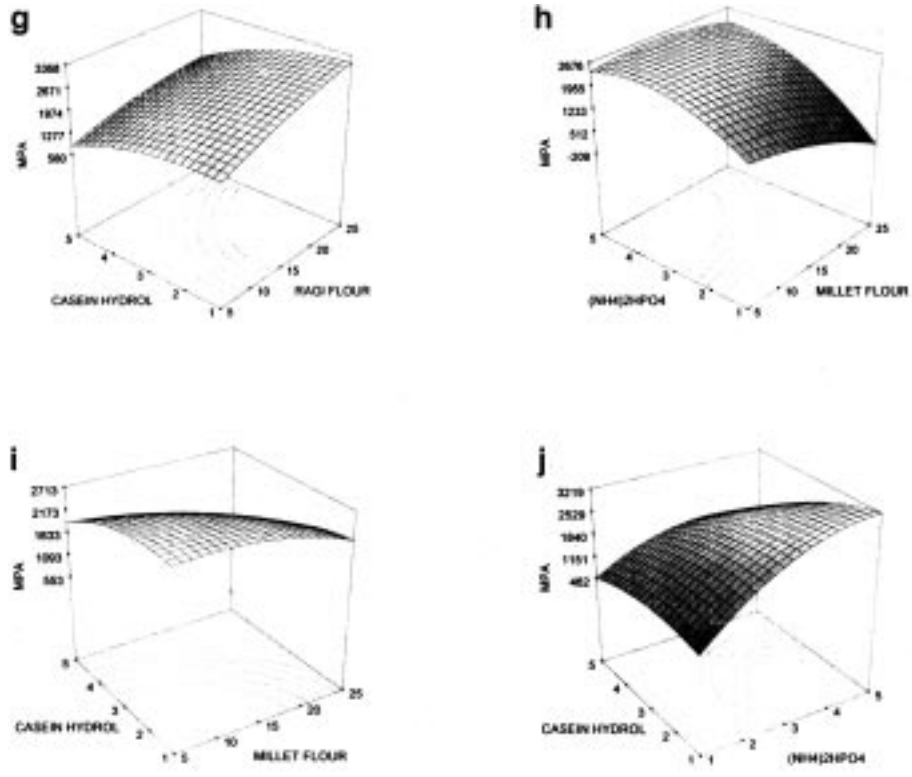

Figure 2 Continued.

further media optimization. The nutrients were studied in different combinations in a full factorial composite rotatable design (CCRD). The values of the coefficients and r^2 for the dependent variable (MPA) are given in Table 4. The r^2 value 0.8268 ensured a satisfactory adjustment of the quadratic model used to the experimental data. The production of MPA may best be predicted by Equation 1.

The three-dimensional response surface curves were plotted to understand the interaction of nutrients and the optimum concentration of each nutrient required for optimum MPA production. A linear effect was observed. With an increase in the concentration of mannitol, MPA titres increased to a maximum at 3% irrespective of the presence of any other nutrient; any further increase in mannitol concentration decreased the production of MPA (Figure 2 a, b, c and d). Thus mannitol at 3% was considered optimum. On the other hand, the production of MPA increased with increased concentrations of ragi flour when tested with other nutrients; maximum titres of MPA were observed with the highest concentration (25%) of ragi flour used in this experiment (Figure 2 a, e, f and g). An optimum concentration of 10% and 4% was observed with millet flour (Figure 2 b, e, h and i) and $(NH_4)_2HPO_4$ (Figure 2 c, f, h and j), respectively. Casein hydrolysate showed a similar trend as millet flour; an increase in production of MPA was observed up to 2% while the titres dropped with further increase in casein hydrolysate concentration (Figure 2 d, g, i and j).

To evaluate the performance of the nutrients in their optimum concentration on the production of MPA, *Penicillium brevi-compactum* DRCC 098 was cultivated in the optimized medium. The strain produced a titre of 3286 ± 235 mg kg^{-1} of wheat bran after 6 days of fermentation and the data were found comparable to the predicted value of 3556 mg kg^{-1} of bran following CCRD analysis. The results from five independent experiments were coincident with the estimated value. Thus, the model was proven to be adequate.

Isolation and purification

Two kilograms of mouldy bran, after 6 days of fermentation, were extracted with 20 litres of commercial grade ethyl acetate. The extract after filtration was concentrated to 3.5 litres under reduced pressure. This was extracted twice with 2 litres of 10% sodium hydroxide (LR grade). The insolubles were removed by filtration and the pH of the filtrate was adjusted to 2 with 50% HCl at room temperature. The precipitate containing MPA was recovered by filtration. The crude product was crystallized as a dark brown product from a mixture of methanol and water (4 : 1). The color impurities were removed by ethyl alcohol. The white residue left, was filtered and recrystallized from aqueous methanol (10%). The process yielded a 60% recovery with a purity of 99%; further improvement in purity level could be achieved by recrystallization. The mycophenolic acid thus obtained, was characterized by means of IR, NMR and mass spectroscopy. The product conforms with the standard MPA obtained from Sigma, USA.

From the present study it appears that MPA could be produced by SSF. The isolation and purification processes are simple. Moreover many of the nutrients required to produce MPA by SSF are from agricultural wastes which would make the process cost effective. This is the first report of MPA production in solid state fermentation.

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