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Cyclosporin Production in Various Solid Substrate Media by *Tolypocladium inflatum* (ATCC 34921)

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Abstract: Cyclosporins are widely produced through submerged fermentations. Solid state fermentation has been used successfully for the production of enzymes and secondary metabolites. Use of different solid substrate medium for the growth and production of cyclosporin by *Tolypocladium inflatum* (ATCC 34921) was carried out to increase the cyclosporin yield and to economize the process of production using solid state fermentation. Locally available and cheaper materials like rice bran, wheat bran, rice husk, wheat husk, rice grit, wheat grit, gingili cake, cottonseed cake, groundnut cake, coconut cake and sunflower cake were used. Among the solid substrates included in the study, wheat bran was found to be supportive both in terms of biomass (22 g kg^{-1}) and cyclosporin production (179 mg kg^{-1}).

Key words: Solid substrates, biomass, cyclosporin, solid substrate fermentation (SSF), submerged fermentation

INTRODUCTION

Cyclosporins are widely used as immuno-suppressants and are reported to have increasing clinical applications (Leung *et al*., 2006). The drug was originally introduced to clinical use for transplantation, a property based upon interference of the agent with lymphokine biosynthesis (Hoppert *et al*., 2001). Cyclosporin plays a major role in reversing multidrug resistance in several types of cancers (Wei *et al*., 2007). While there are numerous reports on the clinical studies, reports on various aspects of production of cyclosporin are limited to submerged aspects of fermentation (Balaraman and Mathews, 1997, 2006), the reports on solid state fermentations are quiet a few (Sekar and Balaraman, 1998; Ramana Murthy *et al*., 1999; Nisha *et al*., 2005). However, SSF is currently used only to a small extent for the enzyme and secondary metabolite production because of process engineering problems (Holker *et al*., 2004).

Tolypocladium inflatum is widely cultivated in the liquid fermentation medium for the production of cyclosporin (Kang *et al*., 2005). Fungi when cultivated on a solid substrate are growing under conditions similar to their natural habitat; they are able to produce the metabolites more efficiently than in SmF (Holker and Lenz, 2005). The present study was focused on the use of different solid substrates for the production of cyclosporin in SSF. Indigenously available cheaper solid substrate materials were included in this study with a main aim to increase the yield of cyclosporin and to minimize the cost of production.

MATERIALS AND METHODS

The fungus *Tolypocladium inflatum* (ATCC 34921) was originally obtained from American Type Culture Collection, Rockville Md, USA in the form of a lyophil and revived on Sabaroud Dextrose medium. The study was conducted for a period of six months starting from May 2007 to October 2007, at the Department of Biotechnology, SRM School of Bioengineering, Chennai, India. MY agar (malt extract 2%, yeast extract 0.4%) was used as the maintenance medium as described by Lee and Agathos (1989) upon revival. Authentic cyclosporin standards (99% purity) were obtained from M/s Sandoz, Hanover, NJ for comparison and evaluation. All the chemicals and biochemicals used in this investigation were of analytical grade purchased from M/s Aldrich Chemicals.

Solid substrates included in this study comprises of wheat bran, rice bran, rice husk, wheat husk, rice grit, wheat grit, gingili cake, cottonseed cake, groundnut cake, coconut cake and sunflower cake. The particle size of solid substrates range between 40 and 160 μm and were added with mineral solution as per the standard procedures (Tengerdy, 1985; Pandey, 1991). Twenty gram of the solid substrates were weighed in 500 mL Erlenmeyer flasks and were sterilized before inoculation.

Conidial suspensions (10^8 mL^{-1}) were prepared in MY broth and 48 h culture was used as the inoculum. The conditions maintained for SSF were 40% initial moisture, at a temperature of 26°C. The flasks were thoroughly

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mixed and incubated in slanting position with periodical mixing and observation for every 24 h. The flasks were incubated up to six weeks for growth and cyclosporin production. All the experiments were carried out in duplicates.

Determination of pH, fungal biomass and sugar estimation were carried out using standard protocols mentioned (Tengerdy, 1985; Pandey, 1991). Cyclosporin was estimated by HPLC after extracting the samples in butyl acetate equipped with C8 column at 60°C with acetonitrile water (70:30) containing 0.01% orthophosphoric acid as mobile phase with a UV detector at 210 nm to confirm the detection of cyclosporin in the fermentation medium using authentic cyclosporin as standard. The retention time and peak area were compared with the authentic standard cyclosporin (Sandimmune, Sandoz).

RESULTS AND DISCUSSION

Effect of solid substrates on biomass production: Among the solid substrates tested for growth of *T. inflatum* (ATCC 34921), wheat bran was observed to support the growth of the fungus starting from first week and the biomass steadily increased with the increase in the incubation period (Table 1). Though rice grit, wheat grit and rice bran showed good visual growth, the biomass was comparatively less than the wheat bran. Although, cottonseed cake and coconut cake supported biomass production, it did not aid in the productivity of the secondary metabolite (Table 2).

Effect of solid substrates on the cyclosporin production: All the solid substrates which showed good growth in terms of biomass did not show cyclosporin productivity. Cyclosporin yield was observed to reach the maximum on the 4th week of incubation and after which it declined (Table 3). Wheat grit and rice grit supported the fungus in terms of biomass 17.8 and 16.7 g kg^{-1}, respectively, however; cyclosporin production was not favored by these substrates (72 and 80 mg kg^{-1}, respectively) (Table 2). Cyclosporin being an intracellular metabolite is released into the medium during the stationary phase after the autolysis of the fungal cell wall. Wheat bran was found to be supportive both in terms of biomass (26.4 g kg^{-1}) and drug production (179 mg kg^{-1}).

A direct comparison between SSF substrates is very difficult due to the different consistencies and

Table 1: Visual growth observations of *Tolypocladium inflatum* (ATCC 34921) on various solid substrates at different periods of incubation

	Incubation period (weeks)					
Solid substrates	1	2	3	4	5	6
Rice bran	+	+	++	++	++	++
Wheat bran	+	++	+++	+++	+++	+++
Rice grit	+	++	+++	+++	+++	+++
Wheat grit	+	++	+++	++	++	++
Rice husk	-	-	+	+	+	+
Wheat husk	-	+	+	+	+	+
Cottonseed cake	-	+	+	+	+	+
Gingili cake	-	-	+	+	+	+
Groundnut cake	-	-	+	+	+	+
Coconut cake	-	+	+	+	+	+
Sunflower cake	-	-	+	+	+	+

+: Growth, ++: Good growth, +++: Affluent growth and -: No growth

Table 2: Effect of various solid substrates on the cyclosporin and biomass production by *Tolypocladium inflatum* (ATCC 34921) after 4 weeks incubation at an initial pH of 5.6 and incubation temperature at 26°C

Substrates used	Final pH	Sugar (%)	Biomass (g kg^{-1})	Cyclosporin (mg kg^{-1})
Rice bran	4.8	2.2	10.4	58
Wheat bran	5.2	1.6	22.4	179
Rice grit	4.9	2.9	16.7	80
Wheat grit	5.0	3.1	17.8	72
Rice husk	4.1	1.8	4.1	34
Wheat husk	4.2	1.9	5.6	29
Cottonseed cake	4.3	3.9	7.3	90
Gingili cake	4.2	6.7	6.5	75
Groundnut cake	4.3	7.1	7.0	81
Coconut cake	4.5	5.6	9.1	80
Sunflower cake	4.5	2.9	9.7	68

Biomass, pH and sugar concentration were determined after 4 week incubation at 26°C, SD = 3.76; SE± 1.5

Table 3: Growth and cyclosporin production on various solid substrates by *Tolypocladium inflatum* (ATCC 34921) at different fermentation periods at 26°C

	Incubation period (weeks)											
	1		2		3		4		5		6	
Substrates used	B	C	B	C	B	C	B	C	B	C	B	C
Rice bran	4.2	Nil	7.0	19	9.9	30	10.4	58	10.1	67	9.9	49
Wheat bran	5.7	Nil	10.5	71	18.6	109	22.4	179	21.8	163	19.6	110
Rice grit	3.6	Nil	9.8	20	14.1	52	16.7	80	17.3	86	14.9	63
Wheat grit	4.5	Nil	9.4	Nil	13.0	28	17.8	72	18.4	95	16.6	47
Rice husk	Ng	ND	Ng	ND	3.2	19	4.1	34	3.7	12	3.6	Nil
Wheat husk	Ng	ND	3.6	Nil	4.1	Nil	5.6	29	7.3	47	9.4	33
Cottonseed cake	Ng	ND	3.0	47	5.4	74	7.3	90	8.6	62	7.9	37
Gingili cake	Ng	ND	Ng	ND	4.0	58	6.5	75	9.9	97	9.1	42
Groundnut cake	Ng	ND	2.9	32	4.8	66	6.7	81	8.7	94	8.8	63
Coconut cake	Ng	ND	3.7	Nil	6.8	29	9.1	80	11.8	101	11.0	71
Sunflower cake	Ng	ND	3.2	Nil	7.0	31	9.7	68	10.1	73	10.4	77

B: Biomass (g kg^{-1}), C: Cyclosporin (mg kg^{-1}), Ng: No growth, ND: Not Determined SD (n = 3) SE±1.6

composition of the substrates selected. The husk and the bran materials were included in this study as they possess the moisture retaining capacity required for the fungus. An earlier study for the production of cyclosporin using wheat bran was attempted (Nisha and Kannan, 2005) while a comparative study with different solid substrates were not made.

Wheat bran is reported to contain high levels of polysaccharide but low levels of nitrogen. Since polysaccharides have much higher moisture absorption potential than lignin, wheat bran is able to retain higher moisture levels (De Souza and Peralta, 2001). Among the various solid substrates tested, wheat bran was observed to be a complete solid medium for the fungal proliferation and cyclosporin production in the SSF process. The particles of wheat bran have the ability to attract and retain the moisture levels mainly because of the hydrophilic functional groups in its organic matter are able to form hydrogen bonds with water molecules (Holker *et al*., 2004). The studies on the production of secondary metabolites (Robinson *et al*., 2001) confirm the use of wheat bran as the best choice of solid substrate both in terms of the biomass and the secondary metabolite production in SSF bioprocess. Use of wheat bran for the production of this drug could economize the bioprocess and thereby the cost of cyclosporin on large-scale production. Further studies on the Scale-up of the bioprocess will help in determining the economy of the wheat bran substrate for cyclosporin production.

REFERENCES

Balaraman K. and N. Mathews, 1997. Process for the preparation of cyclosporin A from *Tolypocladium* species. U.S. Patent No. 5: 656, 459.

Balaraman, K. and N. Mathews, 2006. Optimization of media composition for the production of cyclosporin A by *Tolypocladium* species. Ind. J. Med. Res., 123: 525-530.

De Souza, D.F. and P.M. Peralta, 2001. Production of amylase by *Aspergillus tamari* in solid state fermentation at high initial glucose concentrations. Acta Scientiarum, 23 (2): 599-602.

Holker, U., M. Hofer and J. Lenz, 2004. Biotechnological advantages of laboratory-scale solid state fermentation with fungi. Applied Microbiol. Biotechnol., 64: 175-186.

Holker, U. and J. Lenz, 2005. Solid-state fermentations are there any biotechnical advantages. Curr. Opin. Microbiol., 8 (3): 301-306.

Hoppert, M., C. Gentzch and K. Schorgendrofer, 2001. Structure and localization of cyclosporin synthetase. The key enzyme of cyclosporin biosynthesis in *Tolypocladium inflatum*. Arch. Microbiol., 176 (1): 285-293.

Kang, S.W., S.H. Lee, C.S. Yoon and S.W. Kim, 2005. Conidia production by *Beauveria bassiana* (for the biocontrol of a diamondback moth) during solid-state fermentation in a packed-bed bioreactor. Biotechnol. Lett., 27: 135-139.

Lee, J. and S.N. Agathos, 1989. Effect of amino acids on the production of cyclosporin A by *Tolypocladium inflatum*. Biotechnol. Lett., 11: 77-82.

Leung, P.H., Q.X. Zhang and J.Y. Wu, 2006. Mycelium cultivation, chemical composition and antitumour activity of a *Tolupocladium* sp. Fungus isolated from wild *Cordeyceps sinensis*. J. Applied Microbiol., 101 (2): 275-283.

Nisha, A.K. and N. Kannan, 2005. A comparative study on the production of cyclosporin in submerged and solid-state fermentation process. Ind. J. Applied Microbiol., pp: 95-97.

Nisha, A.K., N. Kannan and A. Michael, 2005. Production of cyclosporin in Solid-State fermentation by *Tolypocladium inflatum*. Ind. J. Applied Microbiol., pp: 57-60.

Pandey, A., 1991. Aspects of fermenter design for Solid-state fermentations. Process Biochem., 26: 355-361.

Ramana Murthy, M.V., E.V.S. Mohan and A.K. Sadhukhan, 1999. Cyclosporin A production by *Tolypocladium inflatum* using solid state fermentation. Process Biochem., 34: 269-280.

Robinson, T., D. Singh and P. Nigam, 2001. Solid state fermentation: A promising technology for secondary metabolite production. Applied Microbiol. Biotechnol., 55 (3): 284-289.

Sekar, C. and K. Balaraman, 1998. Optimization studies on the production of cyclosporin A by solid state fermentation. Bioprocess. Eng., 18: 293-296.

Tengerdy, R.P., 1985. Solid-state fermentations. Trends. Biotechnol., 3: 96-99.

Wei, M., M. Sasmita, G. Katrina, P.M. Jyoti, N. Devki, E.R. Neil, B.A. Jonathan and K. Ashok, 2007. Cyclosporin A and FK506 inhibit IL-12p40 production through the calmdulin/calmodulin-dependent protein kinase-activated phosphoinositide 3-kinase in lipopolysaccharide-stimulated human monocytic cells. J. Biol. Chem., 282 (18): 13351-13362.