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A Novel Medium for the Enhanced Production of Cyclosporin A by *Tolypocladium inflatum* MTCC 557 Using Solid State Fermentation

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Cyclosporin A (CyA) produced by *Tolypocladium inflatum* is a promising drug owing to its immunosuppressive and antifungal activities. From an industrial point of view, the necessity to obtain a suitable and economic medium for higher production of CyA was the aim of this work. The present study evaluated the effect of different fermentation parameters in solid state fermentation, such as selection of solid substrate, hydrolysis of substrates, initial moisture content, supplementation of salts, additional carbon, and nitrogen sources, as well as the inoculum age and size, on production of CyA by *Tolypocladium inflatum* MTCC 557. The fermentation was carried out at 25±2°C for 9 days. A combination of hydrolyzed wheat bran flour and coconut oil cake (1:1) at 70% initial moisture content supported a maximum production of 3,872±156 mg CyA/kg substrate as compared with 792±33 mg/kg substrate before optimization. Furthermore, supplementation of salts, glycerol (1% w/w), and ammonium sulfate (1% w/w) increased the production of CyA to 5,454±75 mg/kg substrate. Inoculation of 5 g of solid substrate with 6 ml of 72-h-old seed culture resulted in a maximum production of 6,480±95 mg CyA/kg substrate.

Keywords: Solid state fermentation, cyclosporin A, wheat bran flour, coconut oil cake, *Tolypocladium inflatum*

Cyclosporin A (CyA), a cyclic undecapeptide antibiotic with immunosuppressive as well as antifungal activities, has markedly increased the success of organ transplantation such as liver and kidney [7]. It suppresses cell-mediated immunity and prevents grafts rejection, and yet leaves the recipient with enough immune activity to combat bacterial infection. It is used as a second-line drug in autoimmune diseases like rheumatoid arthritis, uveitis, bronchial asthma, and inflammatory bowel disease [12, 26]. The organisms known to produce CyA include *Tolypocladium inflatum* [2], *Fusarium solani* [25], *Neocosmospora varinfecta* [16], and *Aspergillus niger* [24]. CyA is reported to be produced by submerged culture fermentation (Smf) [2], static fermentation [5], and solid state fermentation (SSF) [27], and also synthesized enzymically [6]. The maximum reported production of CyA by SSF using *T. inflatum* DRCC 106 is 4,843 mg/kg substrate.

SSF offers advantages of using agro-industrial residues as substrate that usually provides all the nutrients required for growth. It gives higher yields as compared with conventional submerged fermentation, provides a natural habitat for fungal organisms, requires lower capital investment, generates lower volume of polluting effluents, and minimizes contamination problems [19, 21]. In the recent past, SSF has gained importance for the production of several high-value low-volume products like antibiotics [4]. Achieving high titers of enzymes and organic acids by SSF with fungi has been successful [15, 22].

To the best of our knowledge, there are no reports on using a combination of substrates as well as the supplementation of additional salts, carbon, and nitrogen sources for the production of CyA. This paper reports a detailed study on the optimization of CyA production by SSF using *T. inflatum* MTCC 557. In the first step, agricultural wastes and various oil cakes were screened for maximum production of CyA, after which the effect of hydrolysis of starchy substrates, combination of different solid substrates, and the initial moisture content were checked. Furthermore, the effects of additional supplements, such as salts, and additional carbon source and nitrogen source were also investigated.

Materials and Methods

Materials

Glucose, sucrose, maltose, glycerol, yeast extract, agar, malt extract, mycological peptone, casein peptone, ammonium sulfate, sodium nitrate, and urea were procured from Himedia Ltd, Mumbai, India

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Salts like magnesium sulfate, sodium chloride, zinc chloride, manganese chloride, cobaltous chloride, ferric chloride, and zinc sulfate, and solvents like acetonitrile, *n*-butyl acetate, sodium hydroxide, concentrated hydrochloric acid, and sulfuric acid, were purchased from S. D. Fine Chem Ltd. Mumbai, India All solvents used were of AR grade, except acetonitrile, which was of HPLC grade. Standard CyA (authentic sample) was a gift sample through the kind courtesy of RPG Life Sciences Ltd., Mumbai, India Wheat bran flour (WBF), rice bran (RB), millet flour (MF), jowar flour (JF), ragi flour (RF), rice husk (RH), coconut oil cake (COC), sunflower oil cake (SuOC), groundnut oil cake (GOC), flax seed oil cake (FOC), and sesame oil cake (SeOC) were collected from local market. Cottonseed meal (CSM) was a gift sample from the Central Institute for Research on Cotton Technology (CIRCOT), Matunga, Mumbai, India Pall 0.2-μm membrane filter (Ultipor N_{66} Nylon 6, 6 membranes) was purchased from Pall Sciences, Pall Pharmalab Filtration Pvt. Ltd. Mumbai, India.

Microorganisms

Strains of *Tolypocladium inflatum* MTCC 989, *Tolypocladium inflatum* MTCC 557 (indicated as *Beauveria nivea* in the MTCC catalog), and *Tolypocladium inflatum* NCIM 1283 were procured from MTCC, Chandigarh, India and NCIM, Pune, India. *Tolypocladium inflatum* NRRL 18950 was a gift sample from ARS Culture Collection, U.S.A. The cultures were maintained on agar slants containing malt extract 2% and yeast extract 0.4% (MYA), pH 5.4, at 4°C after growing it for 12 days at 24°C. The strains were screened for the maximum production of CyA using SSF.

Preparation of the Seed Inoculum

The organism was subcultured onto a fresh MYA slant and incubated at 25±2°C. After 12 days, to a fully grown slant, 10 ml of sterile saline containing 0.1% Tween 20 was added and mixed well. One ml of this saline containing approximately 10^8–10^9 spores was added to 50 ml of medium composed of malt extract 2%, yeast extract 0.4%, pH 5.4, taken in a 250-ml flask and incubated at 180 rpm for 72 h at 25±2°C. This was used as the seed for SSF.

Fermentation

Five g samples of substrate in total (single substrate or in combination) were placed in 250-ml Erlenmeyer flasks, and distilled water was added in order to produce the required initial moisture content. The flasks were then autoclaved for 20 min at 121°C/15 psi. After cooling the flasks to 29±2°C, 3 ml of seed was added, the contents in the flask were thoroughly mixed, and then the flasks were incubated at 25±2°C. All the experiments were performed in triplicates.

Optimization of Fermentation Conditions

Effect of fermentation time. *T. inflatum* MTCC 557 was tested for the production of CyA at different time intervals between 7 to 14 days.

Evaluation of different substrates. Different solid substrates such as WBF, RB, MF, JF, RF, RH, and CSM and various oil cakes such as COC, SuOC, GOC, FOC, and SeOC were screened for their suitability for the maximum production of CyA. Starchy substrates such as WBF, RB, MF, JF, RF, and RH were hydrolyzed by autoclaving for 20 min at 121°C/15 psi with double volume of hydrochloric and sulfuric acids (0.1, 0.2, and 0.4 N). The excess acid was neutralized with sodium hydroxide, after which the substrate was dried, ground, and passed through #60 sieve and evaluated for production of CyA.

Effect of combination of substrates. Various combinations of hydrolyzed wheat bran flour (HWBF) along with hydrolyzed millet, jowar flours, rice bran, rice husk, and coconut oil cake in 1:1 ratio were evaluated for the maximum production of CyA using *T. inflatum* MTCC 557. The combination of HWBF and COC was evaluated at different ratios. The optimized combination supporting maximum production of CyA was used for further studies.

Effect of initial moisture content. In order to study the effect of moisture content, 3 ml of seed media per 5 g of substrate was maintained constant, while the moisture content was varied between 55% and 80% by adding distilled water before autoclaving. The fermentation was carried out at 25±2°C for 9 days.

Effect of supplementation with salt solution. Three different salt supplements consisting of (% w/w) $FeCl_3$ 0.25; $ZnSO_4$ 0.15 and $CoCl_2$ 0.05 [23]; KH_2PO_4 0.5, KCl 0.25, and $ZnSO_4$ 0.15 [2]; and NaCl 0.1, KH_2PO_4 0.2, $MgSO_4$ 0.1, and $NaNO_3$ 0.5 [29] were evaluated for the production of CyA.

Effect of supplementation of carbon source. Various carbon sources including monosaccharides such as glucose and fructose, disaccharides such as sucrose and maltose, and complex carbon sources such as maltodextrin, starch, and glycerol were assessed as additional carbon sources for the production of CyA using *T. inflatum* MTCC 557. All the carbon sources were evaluated at 1% w/w and supplemented though the moistening agent.

Effect of supplementation of nitrogen source. Different nitrogen sources such as casein peptone, yeast extract, beef extract, bactopeptone, ammonium sulfate, and sodium nitrate were evaluated as additional nitrogen sources for production of CyA. They were added as 1% w/w of solid substrate to the moistening agent. The combination of optimized carbon and nitrogen source was also checked for CyA production using *T. inflatum* MTCC 557.

Effect of initial pH of the supplement. In order to study the effect of pH on CyA production, fermentation experiments were carried out at different initial pH values of supplement solution. The pH was varied between 2–7 with hydrochloric acid or sodium hydroxide.

Effect of slant age, inoculum age, and inoculum size. To study the effect of these parameters, seed culture was inoculated with 1 ml of spore suspension obtained from subcultured culture slants of different time intervals (4–14 days). Solid substrate was inoculated with seed inoculum of different culture ages (24–96 h) at various sizes (1–8 ml) per 5 g of substrate and the fermentation was carried out at 25±2°C for 9 days.

Effect of incubation temperature. This was studied by incubating the flasks at different temperatures from 20 to 45°C.

CyA Extraction and Estimation

The fermented substrate was extracted with 40 ml of butyl acetate at 25±2°C on an orbital shaker at 180 rpm for 24 h. This extract was filtered using Whatman filter paper (No. 1) and then using a Pall 0.2-μm membrane filter (Ultipor N_{66} Nylon 6, 6 membranes) to give a brown coloured extract. One ml of the extract was evaporated under vacuum to dryness. The dried extract was dissolved in an equal volume (1 ml) of HPLC-grade acetonitrile. Twenty μl of sample was analyzed for CyA content using HPLC (Jasko system) fitted with a reverse phase column, Waters Sperisorb ODS (C_{18} octadecyl silane, 250×4.6 mm ID) by the method described by Niederberger *et al.* [17] with slight modification. The mobile phase consisted of acetonitrile and water in the ratio of 70:30 with a flow rate of 1 ml/min. The temperature of the column was maintained at 70°C and the HPLC profile was monitored at 210 nm.

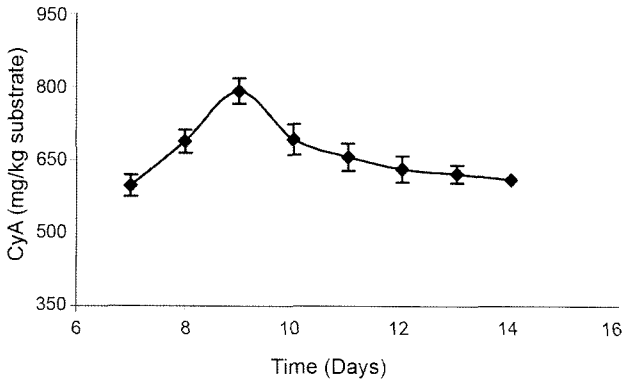

Fig. 1. Effect of fermentation time on production of CyA using *T. inflatum* MTCC 557.

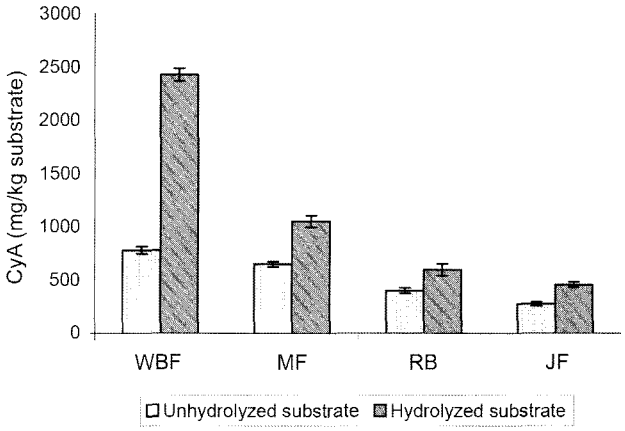

Fig. 2. Effect of hydrolysis on CyA production of various agricultural substrates for CyA production using *T. inflatum* MTCC 557.

Results

Screening of Microbial Strains

Several strains were screened for the maximum production of CyA by using WBF as solid substrate, initial moisture content of 60%, and 3 ml of 72-h-old seed inoculum at 25±2°C for 14 days. *T. inflatum* MTCC 557 gave a maximum production of 674±48 mg/kg WBF, followed by 556±41 mg/kg WBF by *T. inflatum* NRRL 18950, and hence *T. inflatum* MTCC 557 was used for the further optimization studies. *T. inflatum* MTCC 989 and *T. inflatum* NCIM 1283 gave lower titers of CyA.

Effect of Fermentation Time

Three flasks were evaluated every day from day 7 to 14 days to study the effect of fermentation time on CyA production. Maximum production of 792±24 mg/kg WBF CyA was achieved after 9 days, and decreased to 610±18 mg/kg WBF on the 14th day (Fig. 1).

Evaluation of Solid Substrates

Among the agricultural substrates, WBF supported maximum CyA production of 792±33 mg/kg followed by 655±26 mg/kg substrate, 405±24 mg/kg substrate, and 280±20 mg/kg substrate in MF, RB, and JF respectively. All the oil cakes supported the growth of *T. inflatum* MTCC 557, but only COC and SuOC supported the CyA production of 489±59 mg/kg and 376±36 mg/kg substrates, respectively. Hydrolysis of WBF using 0.2N H_2SO_4 resulted in the maximum production of 2,460±120 mg/kg hydrolyzed wheat bran flour (HWBF) (Table 1). Other starchy substrates such as MF, JF, RB, and RH were also hydrolyzed using 0.2 N H_2SO_4 and checked for the CyA production. Hydrolysis of all the substrates increased the production as compared with the non-hydrolyzed states, with HWBF supporting maximum CyA production (Fig. 2). HWBF and COC (1:1) gave a maximum production of 2,918±120 mg/kg combined substrate (Fig. 3). This combination was further evaluated at different ratios (total 5 g) (Table 2), and a 1:1 combination proved to be the best.

Table 1. Effect of hydrolysis of WBF by HCl and H_2SO_4 on production of CyA using *T. inflatum* MTCC 557.

Acid (N)	pH (Supernatant)	CyA (mg/kg WBF)[a]
Control	6.1	785±59
HCl (0.1)	5.7	836±87
HCl (0.2)	5.1	1,295±74
HCl (0.4)	4.3	1,398±35
H_2SO_4 (0.1)	4.7	1,400±80
H_2SO_4 (0.2)	2.4	2,460±120
H_2SO_4 (0.4)	1.8	1,100±78

[a]Results are the mean ± SD of three determinations

Effect of Initial Moisture Content

An initial moisture content of 70% supported maximum production of 3,872±156 mg/kg substrate as compared with 2,607±150 mg/kg and 2,535±124 mg/kg at 55% and 80%, respectively (Fig. 4).

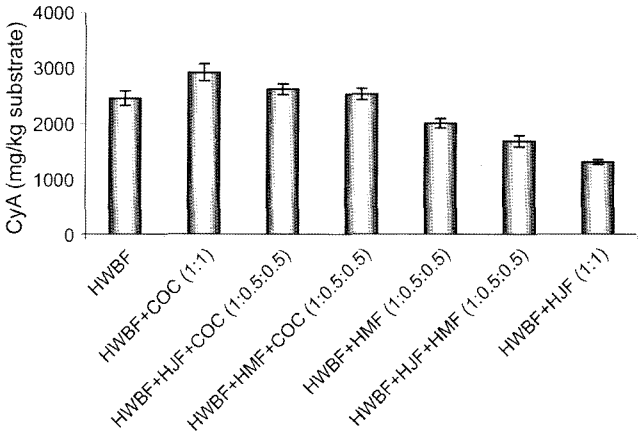

Fig. 3. Effect of combinations of different substrates on CyA production by *T. inflatum* MTCC 557

Table 2. Effect of ratio of HWBF:COC on the production of CyA by *T. inflatum* MTCC 557.

Ratio (HWBF:COC)	CyA (mg/kg substrate)[a]
0:100	489±23
20:80	2,228±95
40:60	2,530±48
50:50	2,956±87
60:40	2,630±56
80:20	2,558±44
100:0	2,333±66

[a]Results are the mean ± SD of three determinations

Effect of Supplementation of Salts, Carbon Source, and Nitrogen Source

Among the salt supplements screened, the one reported by Ramana Murthy *et al.* [23] increased the yield of CyA marginally by 300 mg/kg of substrate. Addition of glycerol resulted in the maximum CyA production of 4,659±58 mg/kg substrate followed by maltodextrin and maltose (Fig. 5). Supplementation of fructose gave only a marginal increase of 4,163±87 mg/kg in CyA production as compared with an unsupplemented control value of 4,060±65 mg/kg The fermentation was carried out using HWBF and COC (1:1) as substrate, 70% of initial moisture content, at 25±2°C for 9 days. Addition of ammonium sulfate resulted in maximum production of 5,014±65 mg/kg followed by 4,858±45 mg/kg bacto-peptone and 4,827±47 mg/kg casein peptone, respectively (Fig. 6). Sodium nitrate increased the CyA production minimally to 4,111±57 mg/kg. The combination of glycerol (1%) and ammonium sulfate (1%) gave a CyA production of 5,454±44 mg/kg.

Effect of Initial pH of the Supplement

Maximum production of CyA (5,848±56 mg/kg substrate) was observed at pH 2 (data not shown). The production decreased at higher initial pH of the supplement. At an initial pH 7, production of 4,423±46 mg/kg substrate was observed.

Effect of Slant Age, Inoculum Age, and Inoculum Size

It was observed that slants incubated for 10 days and above were well sporulated; using such slants for seed inoculation

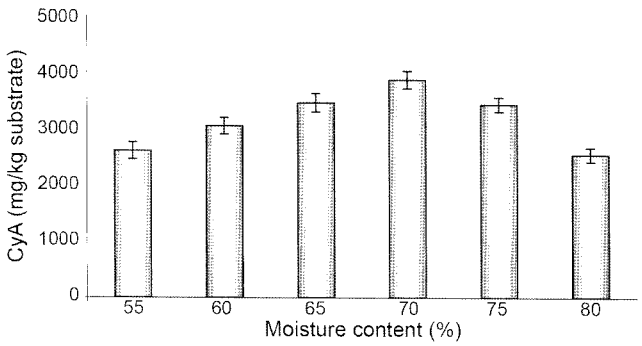

Fig. 4. Effect of initial moisture content on CyA production in SSF by using *T. inflatum* MTCC 557.

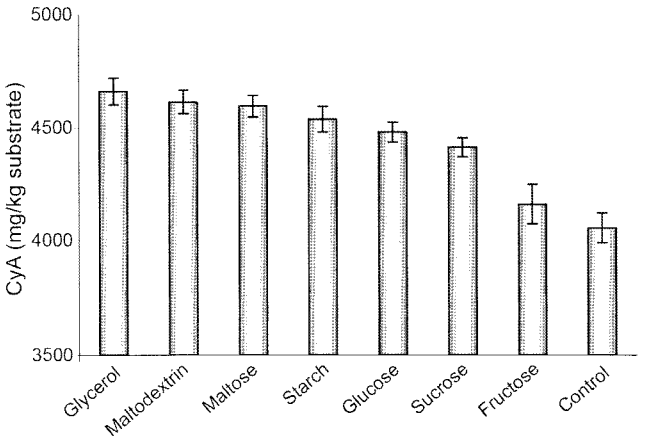

Fig. 5. Effect of additional carbon source supplementation on CyA production in SSF by using *T. inflatum* MTCC 557.
All carbon sources were added at 1%.

resulted in higher CyA production, although the difference in yields was found to be statistically insignificant (Table 3). HWBH and COC (1:1) inoculated with seed culture of different age (24–96 h) and different size (1–8 ml) showed that 6 ml of a 72-h-old seed culture resulted in a maximum CyA production of 6,480±95 mg/kg (data not shown). Increased seed culture age and inoculum size did not increase the yields significantly.

Effect of Incubation Temperature

Experiments carried out to study the effect of temperature on CyA production showed maximum production of CyA at 25°C; further increase in temperature decreased CyA production, and no production was observed at 40°C (data not shown).

DISCUSSION

There are very few reports on the production of CyA by SSF. To the best of our knowledge, the use of a combination

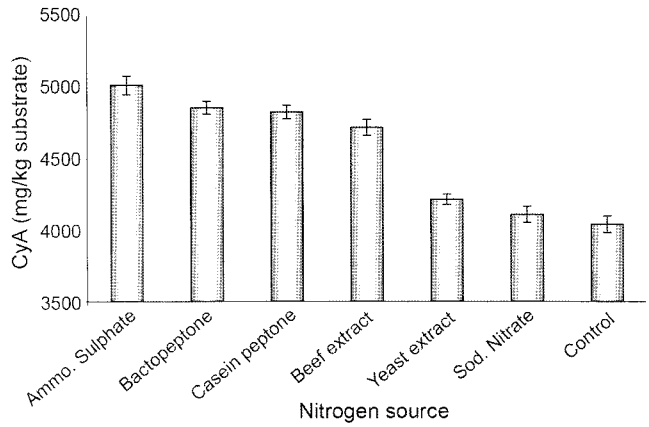

Fig. 6. Effect of additional nitrogen source supplementation on CyA production in SSF by using *T. inflatum* MTCC 557.

Table 3. Effect of slant age on production of CyA by *T. inflatum* MTCC 557.

Slant age (days)	Spore count/ml	CyA (mg/kg substrate)[a]
4	10^2	3,621±25
6	10^4	4,623±36
8	10^7	5,133±54
10	0.2×10^8	5,878±57
12	0.5×10^8	5,902±65
14	0.6×10^9	5,922±48
16	0.8×10^9	5,910±56

[a]Results are the mean ± SD of three determinations

of substrates is not reported in the literature for the production of CyA. *T. inflatum* MTCC 557, giving the maximum production of CyA, was used for optimization studies. Maximum production of CyA was obtained on the 9th day, whereas Ramana Murthy *et al.* [23] and Sekar *et al.* [28] reported maximum CyA productions of 2,600 mg/kg and 1,920 mg/kg bran using *T. inflatum* DRCC 106 and *T. cylindrosporum* F-21 after 10 days.

Among the various substrates screened, WBF supported the maximum production of CyA, which is in accordance with the report by Ramana Murthy *et al.* [23]. Increase in CyA yields after hydrolysis of wheat bran (WB) is also reported by Ramana Murthy *et al.* [23] and Sekar *et al.* [28]. They used 0.2N H_2SO_4 and 0.2N HCl for the hydrolysis of WB, respectively. Ramana Murthy *et al.* [23] reported the maximum production of 1,857±38 mg/kg of hydrolyzed WB as compared with 1,616±85 mg/kg with unhydrolyzed WB. Sekar *et al.* [28] reported that, although the yield of CyA was 160±8 mg/kg of bran cooked with distilled water, it was 1,140±20 mg/kg of hydrolyzed WB. They postulated that the hydrolysis of WB could have released the amino acids, which were incorporated in the CyA molecules. It is possible that the acid hydrolysis of the substrates may have broken down the starch to a readily utilizable form of sugars by the microorganism. COC in combination with HWBF supported the maximum production of CyA. The reason behind COC supporting the production of CyA might be the presence of 25.2% of crude protein that is rich in amino acids such as leucine, valine, and lysine (6%, 5.8%, and 2.8% of the total protein) [18]. These amino acids are reported to enhance the production of CyA [3, 14].

The moisture content has an important role in SSF, although fermentation with relatively no moisture to 30–80% high initial moisture levels are reported [20]. Substrate moisture and water activity (a_w) play an important role in SSF [9, 13]. Ramana Murthy *et al.* [23] reported 70% as the optimal moisture content for maximum CyA production of 1,616±85 mg/kg unhydrolyzed WB using *T. inflatum* DRCC 106. Optimal moisture content depends on the nature of the microorganism and the substrate being used. High moisture content leads to aggregation of substrate particles, poor aeration, and possible anaerobic conditions, whereas very low moisture content restricts the fungal growth. With lower moisture content, the available oxygen in the void volume is sufficient but the water content is not enough to support good metabolic activity and dissipation of the heat generated. This may account for lower production of metabolite and biomass. At very high moisture level, air present in the void volume is replaced by water, thereby decreasing the available oxygen [10, 30].

Increased production of CyA by supplementation of salts could be due to the supporting effect of divalent ions in enhancing the production of CyA by mushrooms [23]. Agathos *et al.* [1] also reported zinc to provide a more complete pattern of glucose utilization, a more stable pH, and higher CyA production. Glycerol and ammonium sulfate supplementation supporting the CyA production was not analogous to the reports by Ramana Murthy *et al.* [23]. They also screened different carbon as well as nitrogen sources as supplements for the production of CyA. They reported that supplementation with 20% millet flour, 10% jowar flour, 0.25% $FeCl_3$, 0.15% $ZnSO_4 \cdot 7H_2O$, and 0.05% $CoCl_2 \cdot 6H_2O$ produced a maximum CyA of 5,043±278 mg/kg bran. Lee and Agathos [14] reported the use of ammonium sulfate as nitrogen source in synthetic media for submerged fermentation. Literature reports on enhanced production of metabolites on supplementation with carbon and nitrogen sources are available. Bussari *et al.* [8] used ammonium oxalate as a supplementary nitrogen source for cephamycin C production using *Streptomyces clavuligerus* NT4. Shaligram *et al.* [29] reported the use of maltose and diammonium hydrogen phosphate as additional carbon and nitrogen sources, respectively, for the production of compactin in SSF using *Penicillium brevicompactum* WA 2315.

The results of initial pH optimization are in agreement with Ramana Murthy *et al.* [23] who reported an initial pH 2 to give better production of CyA (1,857±38 mg/kg WB) as compared with higher pH. Sekar *et al.* [28] also reported a lower initial pH to give higher production, and found the pH of the substrate to increase with the progress of fermentation. Isaac *et al.* [11] reported a higher spore density to give higher production of CyA in submerged fermentation using *T. inflatum* UAMH 2472. Ramana Murthy *et al.* [23] and Sallam *et al.* [24] used 72-h-old seed culture for maximum production of CyA. The activity of cyclosporin synthetase, the enzyme catalyzing the synthesis of CyA synthesis, is reported to be higher at 24°C [31]. This may be the reason for the higher production of CyA at 25°C.

The present study showed SSF to be a good alternative for submerged fermentation for CyA production. Moreover, yields could be substantially increased by optimizing the fermentation conditions and use of supplements. Work on using statistical methods and strain improvements to further improve the yields of CyA are in progress.

Acknowledgments

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