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Lovastatin Production by *Aspergillus terreus* (KM017963) in Submerged and Solid State Fermentation: A Comparative Study

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ABSTRACT

Lovastatin ($C_{24}H_{36}O_5$) is a fungal secondary metabolite that inhibits conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate in cholesterol biosynthesis. Lovastatin producing fungus *Aspergillus terreus* was grown in Solid State Fermentation (SSF) with various agro based wastes and in Submerged Fermentation (SmF) to evaluate the suitable growth medium for maximum production of lovastatin. Eighty three agro based substrates and six different types of SmF media were used for the production. Wheat bran and sprouted wheat were suitable substrates for lovastatin production yielding1.00 mg/G DWS and 1.311 mg/DWS of lovastatin, respectively. None of the SmF medium was found to be suitable for lovastatin production, although all media supported growth of the fungus.

Keywords: Lovastatin. *A. terreus*, SSF, SmF.

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INTRODUCTION

Lovastatin was the first statin drug which was approved by United States Food and Drug Administration (FDA) in the year 1987[1]. It is a potent drug that is used to control increased serum cholesterol level, thereby preventing hypercholesterolemia and associated health issues. It is a competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase which catalyzes a rate-limiting step in cholesterol biosynthesis. Lovastatin is a secondary metabolite produced by a variety of filamentous fungi such as *Monascus ruber, M. purpureus, M. pilosus, M. anka, Penicillium citrinum, Paecilomyces varotii* etc. Currently, *Aspergillus terreus* is employed for commercial production of lovastatin. Several production media have been evaluated for lovastatin production by SmF. Increased yield by supplementation with carbon, nitrogen, amino acids, vitamins, hydrocarbons as inducers have also been well documented[2]. Various types of fermentation media under SmF have been reported[3, 4 ,5]. Solid State Fermentation (SSF) technology is being adapted due to several merits over SmF. They are preferred owing to their low prices, eco-friendly approach, perennial availability, low polluting effluents and relatively easier downstream processing[6]. In general, several cheaply available agro wastes are reportedly being used as substrates for production of pharmaceutically important metabolites**.** Substrates such as black gram, rice straw, pomegranate seeds, molasses, apple waste, rice straw, black gram husk, oil cakes, various varieties of brans etc. have been investigated as potential substrates for lovastatin production[7, 8 ,9, 10]. India being an agricultural nation generates 620 MT of agro based residues which are mainly used as animal fodder and rest disposed, thereby causing environmental issues. The use of SSF as alternative could aid in decreasing the hazards posed by agricultural residues[11]. In the present study, lovastatin producing organism *A. terreus* (KM017963) was grown in several agro based natural substrates to select the best for maximum yield. Several SmF media were also used for lovastatin production and the production efficiency of *A. terreus* was compared between SSF and SmF.

MATERIALS AND METHOD

Submerged Fermentation

Six different nutrient mediua were tested for lovastatin production (Table 1). To 100 ml of production medium, 10µl of spore suspension (10^8spores/ml) of *A. terreus* (KM017963) was added and incubated at 30^{O}C for 8 days at 120 rpm.

Solid State Fermentation

Two grams of each substrate (Table 2) was weighed and moisture content was maintained at

70%. One milliliter of spore suspension (10^8 spores) of *A. terreus* (KM017963) was added to the sterilized substrate and incubated at 28ºC[17].

Table 1: Composition of lovastatin production medium

Media Code	Composition	Quantity(g/L)	Reference
M1	Tomato paste	40	12
	Glucose	12	
	Corn steep liquor	5	
	Trace elements soln.	100ml/L	
M2	Beef extract	1	13
	Peptone	2	
	Glucose	10	
	Yeast extract	1	
	Corn steep liquor	3	
M3	Lactose	120	14
	Yeast extract	1.85	
	KH_2PO_4	2	
	$MgSO_4$	0.52	
	NaCl	0.40	
	$Fe(NO_3)_3$	0.002	
	$ZnSO_4$	.001	
	Biotin	0.04mg	
	Trace element soln.	1 ml	
M4	Dextrose	100	15
	KNO_3	2	
	$NH_4H_2PO_4$	2	
	$MgSO_4$	0.5	
	$CaCl_2$	0.1	
M5	Soluble starch	67.56	16
	Yeast extract	10	
	PEG 2000	2.5	
	KH_2PO_4	2.5	
	$(NH_4)_2PO_4$	1	
M6	Glucose	45	15
	Sodium glutamate	12.5	
	KH_2PO_4	5	
	K_2HPO_4	5	
	$FeSO_4$	0.2	
	$MnSO_4$	0.1	
	$ZnSO_4$	0.2	
	$MgSO_4$	0.1	
	$CaCl_2$	0.02	
	$CuCl_2$	0.005	
	H_3BO_3	0.011	
	$(NH_4)_6PMO_7O_{24}$	0.005	

Table 2: List of solid substrates (agro based materials) used for lovastatin production in *A. terreus*

Sl. No.	Substrate	Sl. No.	Substrate	Sl. No.	Substrate	Sl. No.	Substrate
1	Almond	22	Colocassia	43	Jowar seeds	64	Rice husk
2	Arecanut flower stem	23	Corn cob	44	Litchi peel	65	Tapioca Sago
3	Arecanut seed	24	Corn kernel	45	Marigold	66	Ragi bran
4	Ashgourd seed	25	Corn peel	46	Mosambi skin	67	Raisins
5	*Averrhoa bilimba* fruit	26	Cotton seed cake	47	Mushroom (*Pleorotus* sp.)	68	Saw dust
6	Bamboo Rice husk	27	Cowpea	48	Orange peel	69	Sesame seeds
7	Banana flower	28	Date seed	49	Paddy straw	70	Semolina
8	Banana flower bract	29	Dates (dried)	50	Pasta	71	Soybean powder
9	Banana peel	30	Dates (fresh)	51	Pea peel	72	Spinach
10	Banana stem	31	Egg plant	52	Pomegranate peel	73	Sprouted wheat
11	Barley	32	Flat Beans	53	Pomegranate seed	74	Sugarcane bagasse
12	Beetroot	33	French Beans	54	Potato skin	75	Sunflower cake
13	Black gram	34	Gram husk	55	Processed soya bean (coarse)	76	Spinach
14	Bread (white)	35	Green gram bran	56	Processed soya bean (powdered)	77	Sweet potato
15	Bread (wheat)	36	Green Peas	57	Psyllium husk	78	Tamarind shell
16	Broken Red rice	37	Green Pigeon Peas	58	Pumpkin (fresh)	79	Tapioca peel
17	Cabbage	38	Ground nut cake	59	Pumpkin seeds	80	Tomato (sun dried)
18	Carrot	39	Ground nut shell	60	Ragi bran	81	Urud dhal
19	Chick Peas	40	Guava	61	Red kidney Bean (ground)	82	Wheat bran
20	Coconut cake	41	Jack fruit seeds	62	Red kidney Bean (whole)	83	Wheat husk
21	Coffee husk	42	Jowar husk	63	Red rice (whole)	84	Yam

Extraction of Lovastatin

SmF:

Following incubation of the fungal culture for a predetermined period time, lovastatin was extracted using equal quantity of ethyl acetate to the filtered liquid medium and organic phase (ethyl acetate) used for quantitative assay of lovastatin[15].

SSF:

After eight days of incubation, the solid substrate along with the mycelial mat was dried at 40ºC for 24 hrs, crushed and extracted with 10ml ethyl acetate by shaking at 180 rpm for 2 hrs followed by filtration through Whatman No. 1 filter paper. To 1ml of extract, 1ml of 1% trifluroacetic acid was added and incubated for 10 minutes (lactonization of hydroxyl acid form of lovastatin). The filtrate was then spotted onto Thin Layer Chromatography (TLC) for detecting the presence of lovastatin in crude extract[17].

ANALYSIS OF LOVASTATIN

Thin Layer Chromatography (TLC)

The extracted organic phase was concentrated to about 50µl using a block heater adjusted to 45ºC, applied to a heat activated 20 × 20cm silica gel TLC plates. Three different solvent systems were used to detect the presence of lovastatin crude extract namely, Dichloromethane: Ethyl acetate (70:30). The plate was observed under a hand-held UV lamp (254nm) after developing three times in the same mobile phase and stained with iodine vapour. For each TLC run, lovastatin authentic standard (Sigma-Mevinolin M2147) was also included for R_f value comparison and confirmation[18].

High Performance Liquid Chromatography (HPLC)

For HPLC analysis, a Shimadzu Liquid Chromatography model LC-10 withC-18 column (250mm x 4.6mm micro metre) and diode array detector was used. Acetonitrile and water acidified with 0.1% phosphoric acid (60:40 v/v) was used as mobile phase. The eluent flow rate was maintained at 1.5ml per minute and detection carried out at 238nm with injection volume of 20 µl. The production of lovastatin is expressed in mg/G Dry Weight Substrate (DWS)[19]. The yield of lovastatin was calculated according to the published method[20]. Mevinolin (M2147) (Sigma-Aldrich, Germany) was used as standard.

RESULTS AND DISCUSSION

In recent days, Solid State Fermentation (SSF) appears the method of choice for any metabolite production as it provides better process control; higher yield besides several other beneficial aspects[21]. For commercial purpose, production of antibiotics and other secondary metabolites are generally achieved by SSF. We have isolated a lovastatin producing soil fungus, *A. terreus* (KM017963) using wheat bran as a substrate with a yield of 1.00 mg/G DWS[6]. In order to achieve better productivity, in the present study, we utilized several agro based substrates including agro wastes which are mainly used as fodder as sole source of substrates for the growth of *A. terreus* (KM017963) and production of lovastatin. In parallel, we used different types of liquid media to select the best medium either as solid or in dissolved condition for the production of lovastatin by *A. terreus* (KM017963). In total, we used 83 naturally available agro based substrates as solid substrates. Out of which, the highest yield was obtained with sprouted wheat (1.311 mg/G DWS) followed by wheat bran (1.00 mg/g DWS). Substrates such as potato peel, date seed, banana flower bract, semolina did not support the production of lovastatin. Other substrates such as banana peel, paddy straw, ground nut cake, coconut oil cake also could not act as a suitable substrate neither for the growth of the organism nor for the production of lovastatin as the lovastatin produced was below the base level. There are several reports available that solid substrates such as black gram husk (12.63 mg/g), green gram husk (4.8 mg/g), orange peel (3.4 mg/g), rice bran (9.2 mg/g) etc are suitable substrates for the increased yield of lovastatin [21, 22, 23]. However, the results from our study indicated that these reported substrates, although support the growth of *A. terreus*, do not favour lovastatin production (Table 3). Out of the six liquid media tested under submerged condition (SmF) for lovastatin production, only two media i.e M2 (0.012 µg/L) and M3 (0.025µg/L), produced low but detectable levels of lovastatin even after extended growth period. Glucose and beef extract as components of synthetic medium of M2 had no positive influence on lovastatin production in *A. terreus* (KM017963) although glucose is reported to be the best carbon source under submerged growth condition[2, 4]. Slowly degradable carbon source like lactose is preferred for lovastatin production in *A. terreus* (ATCC20542)[24]. However, in our study, the medium M3 which has lactose as carbon source and yeast extract as nitrogen source did not influence the production of Lovastatin. The difference in the lovastatin production level under different growth conditions ie SSF and SmF although is been debated by many researchers and possible reasons are given at biochemical level[3]; a comparative study of the same was studied at genetical level[25]. Their study reported that, higher lovastatin production in SSF is attributed to enhanced transcriptional rates of biosynthetic genes *lov*E and *lov*F resulting in yield increase by 4.6 fold and 2 fold, respectively. The *lov*E and *lov*F transcript accumulation was 20 and 6 fold lower than in SSF when a liquid medium (SmF) of identical concentration when used. Expression of *lov*E and *lov*F genes was detected from day 1 in SSF when compared to SmF where gene transcription initiated from day 3 only. The comparative work on lovastatin production established the advantage of SSF with a 30 fold yield increase

(9.56 mg/g and 9.7 mg/g) as against SmF (0.276 and 0.236 mg/ml) in two strains of *A. terreus*[26]. In the present work, we made an attempt to screen several agro based materials as solid substrates for the production of lovastatin by *A. terreus* (KM017963) as there are supporting evidence from the previously published reports that various types of solid substrates including wheat bran can enhance the yield of lovastatin in *A. terreus*[7, 17, 27]. Our results categorically conclude that wheat based materials such as wheat bran and sprouted wheat are the only two suitable substrates for the better yield of lovastatin, among the 83 substrates tested. It is interesting to note that whole wheat as such could not influence the production as compared to the sprouted wheat. This could be due to the metabolic changes during sprouting, although not much difference in the yield of lovastatin was observed when compared with wheat bran[28]. In general, wheat bran is a source of protein, fat, crude fibre, cellulose, pentoses, vitamin (E), minerals and certain phytochemicals[29, 30, 31] and considered as suitable substrate for commercial production of lovastatin[7, 17, 27].

Sl. No.	Substrate	Lovastatin yield (mg/G DWS)
61	Raisins	NG
62	Red kidney Bean (ground)	UD
63	Red kidney Bean (whole)	0.064
64	Red rice (whole)	0.330
65	Rice husk	UD*
66	Tapioca Sago	UD*
67	Ragi bran	UD*
68	Raisins	NG
69	Saw dust	UD*
70	Sesame seeds	UD*
71	Semolina	0.200
72	Soyabean powder	UD
73	Spinach	UD
74	Sprouted wheat	1.311
75	Sugarcane bagasse	UD*
76	Sunflower cake	UD
77	Sweet potato	UD*
78	Tamarind shell	UD*
79	Tapioca peel	UD
80	Tomato (sun dried)	UD
81	Black gram	NG
82	Wheat bran	1.00
83	Wheat husk	UD
84	Yam	UD

*UD: Undetectable level; *NG: No Growth.

Table 3: Lovastatin yield by *A. terreus* grown in various solid substrates under Solid State Fermentation (SSF)

Sl. No.	Substrate	Lovastatin yield (mg/G DWS)	Sl. No.	Substrate	Lovastatin yield (mg/G DWS)	Sl. No.	Substrate	Lovastatin yield (mg/G DWS)
1	Almond	UD	21	Coffee husk	UD*	41	Jack fruit seeds	UD
2	Arecanut flower stem	0.182	22	Colocassia	UD	42	Jowar husk	0.012
3	Arecanut seed	0.225	23	Corn cob	UD*	43	Jowar seeds	0.091
4	Ashgourd seed	UD*	24	Corn kernel	0.128	44	Litchi peel	UD
5	*Averrhoabilimba*fruit	UD*	25	Corn peel	UD*	45	Marigold	UD
6	Bamboo Rice husk	NG	26	Cotton seed cake	UD	46	Mosambi skin	UD*
7	Banana flower	UD	27	Cowpea	UD	47	Mushroom (*Pleorotus* sp.)	UD
8	Banana flower bract	0.169	28	Date seed	0.223	48	Orange peel	UD*
9	Banana peel	UD	29	Dates (dried)	NG	49	Paddy straw	NG
10	Banana stem	UD	30	Dates (fresh)	NG	50	Pasta	UD
11	Barley	0.549	31	Egg plant	UD	51	Pea peel	UD
12	Beetroot	NG	32	Flat Beans	UD	52	Pomegranate peel	UD*
13	Black gram	UD	33	French Beans	UD	53	Pomegranate seed	UD
14	Bread (white)	UD	34	Gram husk	UD*	54	Potato skin	UD
15	Bread (wheat)	UD	35	Green gram husk	UD*	55	Processed soya bean (coarse)	0.144
16	Broken Red rice	0.001	36	Green Peas	UD	56	Processed soya bean (powdered)	UD
17	Cabbage	NG	37	Green Pigeon Peas	UD	57	Psyllium husk	UD
18	Carrot	NG	38	Ground nut cake	UD*	58	Pumpkin (fresh)	NG
19	Chick Peas	UD	39	Ground nut shell	UD*	59	Pumpkin seeds	UD
20	Coconut cake	UD	40	Guava	UD	60	Ragi bran	UD

*UD: Undetectable level; *NG: No Growth

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