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ISOLATION AND CHARACTERIZATION OF LOVASTATIN PRODUCING FOOD GRADE FUNGI FROM ORIENTAL FOODS

Upendra R. S[1]*, Pratima Khandelwal[2], Z. R. Amiri[3], Aparna. S[4], Archana. C[4] and Ashwathi.M[4]

[1]Sr. Assistant Professor, [2]Prof & Head, Dept. of Biotechnology, New Horizon College of Engineering, Marathahalli, Bangalore, Karnataka, India.

[3]Assistant Professor, Dept. of Food Sci. & Tech., Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

[4]BE BT alumni (2009-2013), New Horizon College of Engineering, Marathahalli, Bangalore, Karnataka, India.

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***Correspondence for Author**
Upendra R. S
Sr. Assistant Professor, Dept. of Biotechnology, New Horizon College of Engineering, Ring Road, Bellandur Post, Marathahalli, Bangalore - 560103, Karnataka, India

ABSTRACT

Traditional oriental fermented foods namely soy sauce, miso, vinegar, red rice vinegar, pickled tofu and Ang-kak were becoming increasingly popular in different parts of India and shall be used for isolation of hypolipidemic agent, lovastatin producing representative fungi. Existing food grade (GRAS) fungi, *Monascus sp* were known to produce lovastatin through SSF. Further exploration of food grade fungi from indigenous and traditional oriental fermented food products shall augment such sources and lead to better and safe food product development. Such a strategy shall give benefits to the elderly and the concept of preventive health care shall take envisaged shape. With this objective the present investigation focus on isolation and microbiological characterization of the lovastatin producing GRAS filamentous fungi from various traditional oriental fermented food samples obtained from India and China. Initially food grade fungal cultures were isolated by serial dilution technique and morphological characteristic of the isolated fungal cultures was studied on Potato dextrose agar (PDA) medium. Microscopic confirmation of isolated fungal cultures were performed using light microscopy. Further, microbiologically characterized fungal cultures were tested on a novel substrate combination of rice and barley using Solid state fermentation (SSF) process for the production of lovastatin. At the end of the SSF process the

presence of lovastatin in the prepared samples from the end product was analysed qualitatively using Thin Layer Chromatography and ATR-FTIR. The yield of lovastatin was quantified using UV spectroscopic analysis at 238 nm using pure drug lovastatin as a standard (Biocon, India). Present study isolated one *Monascus purpureus* and three *Monascus ruber* fungal cultures and concluded that all the four characterized fungal cultures were found to be positive for lovastatin production.

KEYWORDS: *Monascus Species,* Traditional oriental fermented foods, Lovastatin, Solid state fermentation and ATR-FTIR.

INTRODUCTION

Lovastatin is a naturally occurring molecule found in food such as red yeast rice, oyster mushroom. [1] *Aspergillus terreus*, *Aspergillus oryzae* and *Monascus sp* are known to produce cholesterol reducing drug- lovastatin**.** Increased cholesterol levels have been associated with cardiovascular diseases (CVD). [2] The WHO estimated that 17.3 million lives were lost in 2008 and an expected 23.6 million people will die of cardiovascular diseases (CVD) by the year 2030. [3] Lovastatin prevents the occurrence of CVD. [4] Lovastatin exhibit action beyond lipid-lowering activity in the prevention of hypercholesterolemia, Parkinson's, Alzheimer's disease, atherosclerosis. It also acts as anti-inflammatory agent and augments bone regeneration. [5] The *Monascus* species is a Chinese traditional fermentation fungus used on food for over thousands of years in China, and its special effects and application on food were recorded in ancient Chinese records. *Monascus* species produce a variety of secondary metabolites of pharmaceutical interest including cholesterol lowering agent such as lovastatin. [6] *Monascus* species are best known for their use in fermented Asian foods such as rice wine and red rice (also known as ang-kak and beni-koji) and as a food colorant. Monascus purpureus is a red mold species which can be cultivated on starch containing substrates. Young colonies of *Monascus ruber* on potato dextrose agar (PDA) were floccose and white with pink tinges and they produce reddish to brown pigments that diffuse into the medium. With age the self-fertile colonies turn brown to orange brown. **[7]**

Ang-kak, a traditional Chinese functional food produced by solid state fermentation of cooked non-glutinous rice with *Monascus* sp., contains different high value secondary metabolites lovastatin, γ-aminobutyric acids (GABA), monascodilone, monascorubramine, monascin, ankaflavin, rubropunctatin.[8,9,10,11] Co-culture of *Monascus purpureus* and *Monascus ruber* was used to produce red mold rice by solid-state fermentation. [12] Fermented

rice (red mold rice) has been found to reduce the serum total cholesterol and triglyceride due to the presence of lovastatin. Further exploration of food grade fungi from indigenous and traditional food products shall augment such sources and lead to better and safe food product development. Such a strategy shall give benefits to the elderly and the concept of preventive health care shall take envisaged shape. In India, most of the traditional fermented foods and beverages are yet to be investigated as only a few common fermented foods such as *idli, dosa, dahi* etc., have been studied so far. [13]

With this lacuna, the present investigation is aimed at the isolation and microbiological characterization of the lovastatin producing GRAS filamentous fungi from various traditional oriental fermented food samples obtained from India and China. Initially food grade fungal cultures were isolated by serial dilution technique and morphological characteristic of the isolated fungal cultures were studied on Potato dextrose agar medium. Microscopic confirmation of these isolated cultures were performed using light microscopy. Further, microbiologically characterized fungal cultures were tested on a novel substrate combination of rice and barley using Solid state fermentation (SSF) process for the production of lovastatin. At the end of the SSF process the presence of lovastatin in the prepared samples was analysed qualitatively using Thin Layer Chromatography and ATR-FTIR. The yield of lovastatin was quantified using UV spectroscopic analysis at 238 nm using pure drug lovastatin as a standard (Biocon, India). Present study isolated one *Monascus purpureus* and three *Monascus ruber* fungal cultures and all the organisms found positive for lovastatin production.

MATERIALS AND METHODS

All the chemicals and reagents used in this study were of Analytical Grade (Merck and Qualigens).

Collection of Samples

Traditional oriental fermented foods namely soy sauce, pickled tofu and Ang-kak were collected in a sterile air tight polythene bag from the restaurants of china, India and the sample type, time, date and the place of collection were recorded. The collected samples were stored at 4^0C for further isolation process.

Isolation of food grade fungi by Standard Microbiological Techniques

From the collected traditional oriental fermented foods samples, food grade fungal cultures were isolated on PDA plates using the simplest and most widely used method for culturing, serial Dilution technique. The sample is subjected to serial successive dilutions in sterile saline solution, so that the concentration of the microbes gradually decreases, food grade fungi form these dilutions were isolated by spread plate technique on PDA plates which were incubated at 28^{o}C for 5-7 days. [14]

Microbiological Characterization

After incubation period the colonies obtained were subcultured (In biology a subculture is a new cell or microbiological culture made by transferring some or all cells from a previous culture to fresh growth medium) on PDA slants to obtain pure cultures. Morphological properties namely colony color, shape, size, margins elevation and growth rate, and microscopic properties such as conidial head, conidiophores, vesicle and conidia were studied for isolated pure fungal cultures. [15]

Solid State Fermentation

The spores were collected from the characterized food grade fungal cultures by single spore isolation technique using 2% Tween-20 solution and diluted to 5.7×10^{6} spores/ml. Spore counting was carried out using a hemocytometer. 7.5 ml of collected spores were added to 40 g of novel production medium (Composition: barley 20g/kg, rice 20g/kg, malt extract 10 g/kg, dextrose 40 g/kg, $MnSO_4.H_2O$ 5g/kg, $MgSO_4.7H_2O$ g/kg and sodium acetate 0.3 g/kg), pH6.5 and moisture content was maintained at 65% (w/v). The production medium was incubated for 15 days at room temperature for lovastatin yield under solidstate state fermentation process (SSF). [16]

Downstream Processing of Lovastatin

After 15 days of fermentation process, the fermented solid substrate matter was dried at 60^{o}C for 1hr. 1g of dried fermented matter was extracted with 10ml of ethyl acetate in a sterile 100 ml conical by keeping flask in shaker incubator for 1 hrs at 25^{o}C and 180 rpm. Supernatant which contains lovastatin was collected for the qualitative and quantitative analysis. [17]

Qualitative Analysis by Thin Layer Chromatography (TLC)

TLC plate was pre-activated in hot air oven at 120°c for 1hour. Standard lovastatin and sample were spotted on the pre-activated TLC plate and allowed it to dry. Chromatogram was

developed by running three times in dichloromethane and ethyl acetate (85:15, v/v) mobile phase. For each TLC run, lovastatin standards and samples were applied for Rf comparison. Plates were observed under a hand-held UV lamp (254 nm) (or) yellow spots were developed in TLC plate using 4-5 crystals of iodine. [18]

ATR-FTIR Analysis of Lovastatin

Final confirmation of lovastatin in the prepared extracts was done using FTIR/Diamond ATR, Model: FTIR-8400S, Brand Name: Shimadzu. ATR was fitted with a single bounce diamond at 45˚ internally reflected incident light providing a sampling area of 1 mm in diameter with a sampling depth of several microns. A small amount of the sample was directly placed on the diamond disk and liquid sample kept in liquid sample holder. Sample was scanned for absorbance over the range from 4000 to 400 wave numbers (cm-1) at solution of 1 cm-1. [19]

UV Spectrophotometric Analysis of Lovastatin

Prepared sample were analyzed qualitatively for the presence of lovastatin at different nm (210 nm – 350nm), subsequently lovastatin was detected and estimated at 238 nm, using pure lovastatin (Biocon laboratories, Bangalore, India) as a standard in UV/Visible spectrophotometer. (Shimadzu, Model no UV-2450 and Software UV-probe 2.21)[20].

RESULTS AND DISCUSSION

Collection of Samples

Collected traditional oriental food samples for the isolation process of food grade fungi were given in Table 1.

Table. 1. Collected traditional oriental food samples for fungal isolation

S.no	Natural sample type	Sample code	Country
1.	Soy sauce,	SSCH1	China
		SSCH2	China
		SSIN1	India
		SSIN2	India
2.	Pickled tofu	PTCH1	China
		PTCH2	China
		PTIN1	India
		PTIN2	India
3.	Ang-kak	AKCH1	China
		AKCH2	China
		AKIN1	India
		AKIN2	India

Isolation and Microbiological characterization of fungal isolates

In the present investigation, four fungal cultures were isolated from oriental fermented food samples on PDA medium. They were characterized using standard microbiological methods such as morphological properties (colony color, Shape, size, margins elevation and growth rate) (Fig 1) and microscopic properties (conidial head, conidiophores, vesicle and conidia) (Fig 2). Characterized and identified fungal cultures were maintained in pure culture form on PDA slants and stored at 4^{o}C.

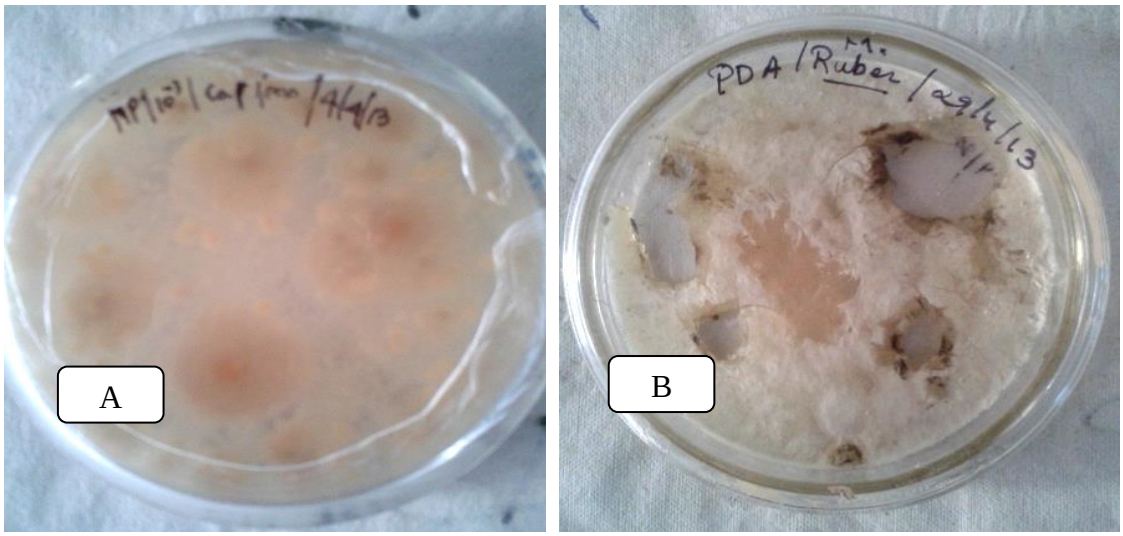

Fig 1. Isolated and Identified fungal cultures, 1[A]. *Monascus purpureus* PTIN2 showing an orangish pigmentation with velvety texture, 1[B] *Monascus ruber* AKIN1 showing white colonies with an orange pigmentation.

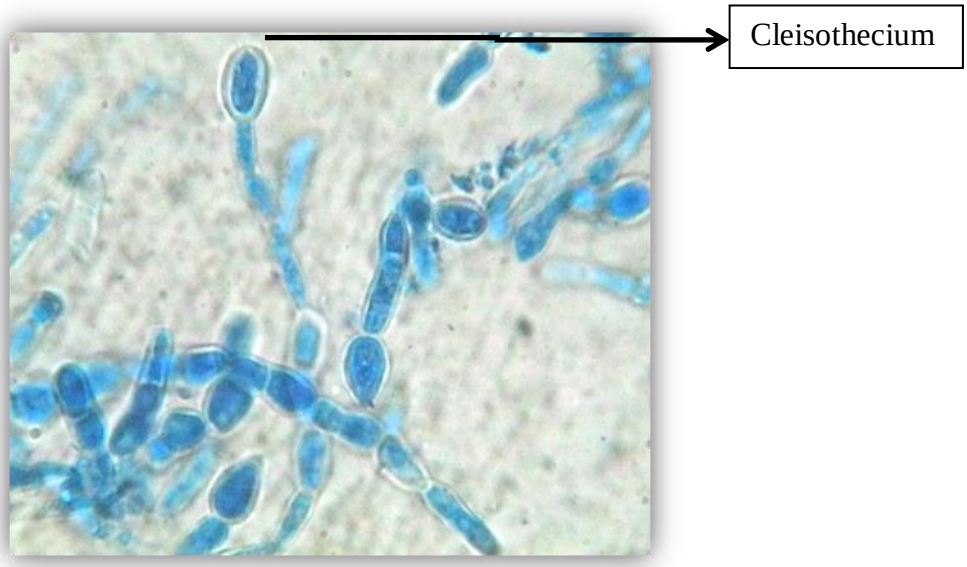

Fig.2. Light microscopy (100 X) of *Monascus purpureus with* Cleisothecium

Solid State Fermentation process and Downstremprocessing

SSF was performed on a novel substrate mixture of rice and barley Fig.3A. At the end of the 15 days of incubation DSP was performed and extracted lovastatin was represented in Fig.3B.

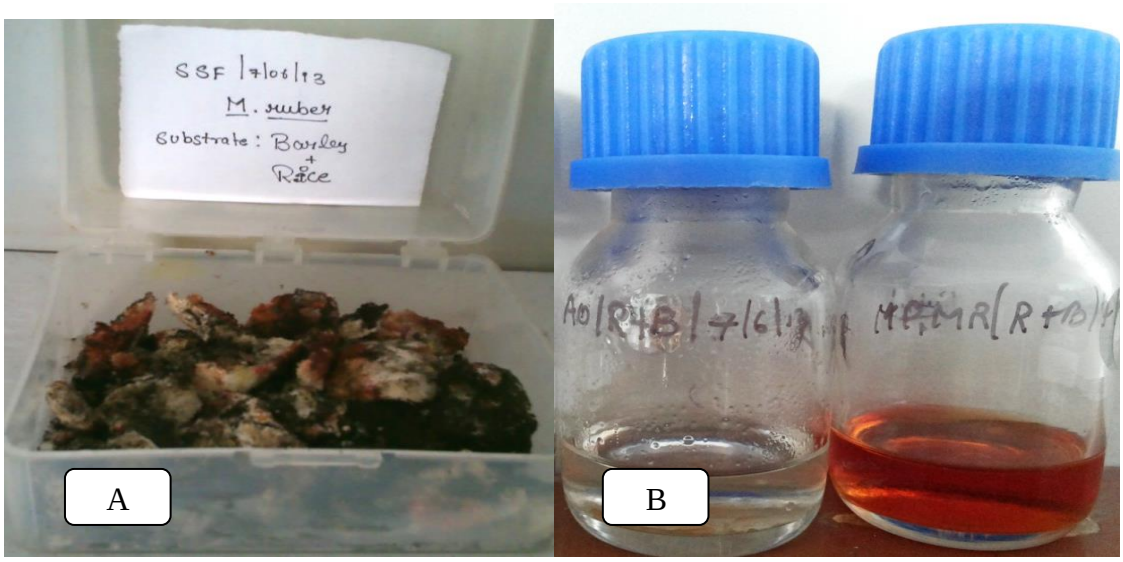

Fig 3. SSF and DSP of lovastatin [3A]. SSF novel substrate (rice and barley). [3B] Extracted lovastatin from novel substrate.

Qualitative Analysis by Thin Layer Chromatography (TLC)

In the thin layer chromatography it was observed that, both commercial lovastatin and the sample spots had shown a same Rf value which implies the qualitative determination of lovastatin in the extracts (Table 2).

Table 2. Rf values of different lovastatin extracts and standard lovastatin

S.no	Sample Type	Rf Value
1	Lovastatin standard 1	0.51
2	*Monascus purpureus* PTIN2 (Rice and Barley)	0.49
3	*Monascus ruber* AKIN1 (Rice and Barley)	0.51
4	Lovastatin Standard 2	0.49

ATR-FTIR Analysis of SSF extracts (lovastatin)

The ATR-FTIR spectra of *Monascus ruber* AKIN1 SSF extract (Lovastatin) was shown in (Fig.4). The spectrum presented characteristic peaks at 1730, 1712, 1703 cm-1 (lactone and ester carbonyl stretch), 1380 cm-1 (methyl symmetric bend), 1357 cm-1 (methyl symmetric bend respectively), 1178.55 cm-1 (lactone C-O-C asymmetric bend), 1080.17 cm-1(lactone CC symmetric bend), 1030.02 cm-1 (ester C-O-C symmetric bend), 895.00 cm-1 (alcohol C-

OH stretch) and 740.00 cm-1(benzene strong) and 692.47 cm-1(C-H, cis-disubstituted alkenes strong)and confirm the presence of lovastatin in the samples. Lovastatin containing the lactone ring gives characteristic peak at 1725, 1711, 1700 cm-1.

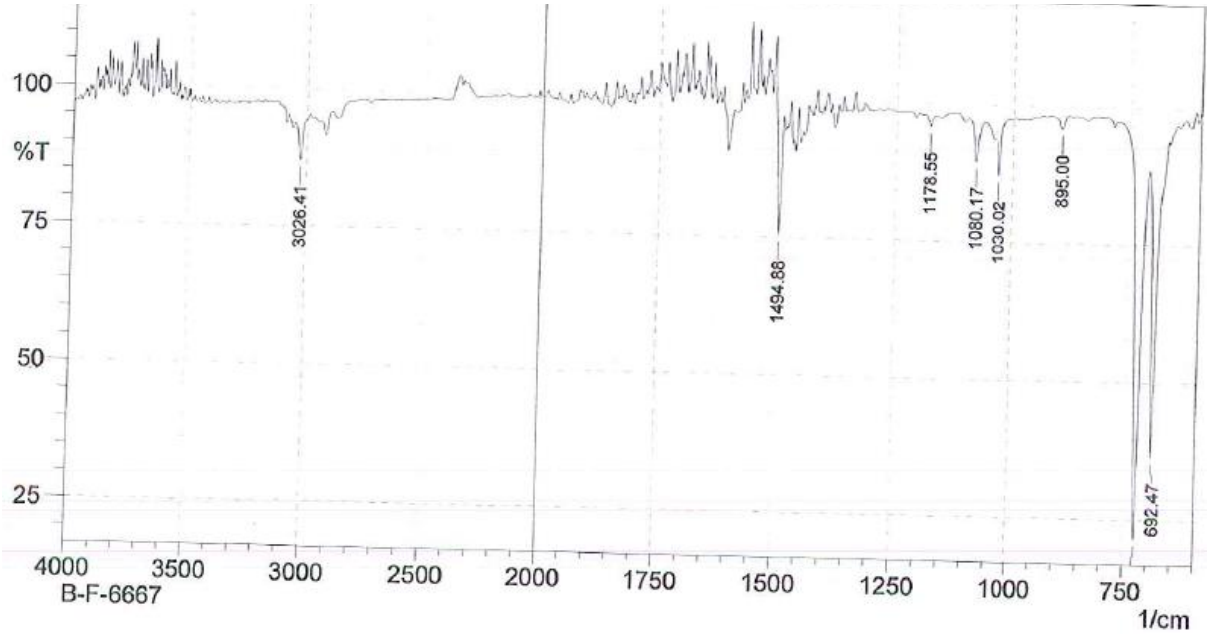

Fig 4: ATR-FTIR analysis of *Monascus ruber* AKIN1 SSF lovastatin extracts

UV Spectrophotometric Analysis of lovastatin SmF samples

The quantitative estimation of lovastatin was done spectrophotometrically at 238 nm. Yields of lovastatin of different culture were presented in Table 3.

Table 3-Isolated fungal culture and their lovastatin yield.

S.no	Sample type/ code	Media/ Dilution	Fungal culture	Yields of lovastatin µg/g dry matter
1.	Pickled to fu PTIN 2	PDA/10^{-4}	*Monascus purpureus*	20
2.	Ang-kak AKCH1	PDA/10^{-3}	*Monascus ruber*	25
3.	Ang-kak AKIN1	PDA/10^{-4}	*Monascus ruber*	50
4.	Ang-kak AKIN2	PDA/10^{-5}	*Monascus ruber*	35

CONCLUSION

The present investigation screened various traditional oriental fermented food samples obtained from India and China for lovastatin producing GRAS filamentous fungi. The isolated fungal cultures were microbiologically characterized and tested on a novel substrate a combination of rice and barley using solid state fermentation (SSF) process for the production of lovastatin. At the end of the SSF process the presence of lovastatin in the prepared samples was analyzed qualitatively using Thin Layer Chromatography and ATR-

FTIR. The yield of lovastatin was quantified using UV spectroscopic analysis at 238 nm using pure drug lovastatin as a standard (Biocon, India). Present study isolated one *Monascus purpureus* and three *Monascus ruber* fungal cultures and concluded that, all the isolated fungal cultures were found to be positive for lovastatin production.

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