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PROUDUCTON OF RED YEAST RICE BY *Monascus purpureus*

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ABSTRACT

Different cultural media were employed to study their effects on the production of red yeast rice (R.Y.R) with high concentration of lovastatin by *Monascus purpureus* . A modified medium containing 200 g sterile local rice, 4 g mannose, 2.5 g monosodium glutamate and 2 g yeast extract at pH 5.0 was inoculated by 25 ml of a 7-day culture of the above fungus and incubated statically at 30 °C for 72 hours, then incubated at 25 °C for 10 days with environmental humidity being around 65 % using these conditions 0.2365 % of lovastatin was produced. The amount of dry R.Y.R. produced was 113 g from 200 g rice, which contains 1.419 mg lovastatin in 600 mg dry R.Y.R.

Keywords : Red yeast rice , Lovastatin production , *Monascus purpureus* , Fermentation , Medium optimization .

INTRODUCTION

Monascus purpureus is a red mold species which may be cultivated on starch containing substrates. The solid state fermentation of rice by *Monascus* has a long tradition in East Asian countries which dates back at least to the first century A.D. Red Yeast Rice (R.Y.R.) is used as food or food additives. R.Y.R., an Asian dietry staple made by fermenting yeast (*Monascus purpureus*) on rice, is rapidly gaining recognition as a cholesterol lowering agent. It is used as coloring and flavoring agents and also reduces total cholesterol and hyperlipidemia. Other exciting applications for R.Y.R. are suggested by recent discoveries that lovastatin and other statin drugs may be useful for treating or preventing cancer, osteoporosis, stroke, Alzheimer`s disease and other dementias, and macular degeneration, (Ozlem Erdogrul and Sebile Azirak , 2004).

Response surface methodology (RSM) was employed to study the effect of culture medium on the production of lovastatin in mixed solid-liquid state (or submerged) cultures by *Monascus ruber* , (Yaw-Nan Chang *et al* , 2002) .

A higher producer of ascospores and pigments, *Monascus* strain TTWMB 6042 , was used to study regulation of pigment production by nutrients . They found that the formation of red pigments in this strain was strongly stimulated by monosodium glutamate (MSG) as the sole nitrogen source and an initial pH 5.5 , (Tzann and Arnold , 1991) .

One hundred and ten fungal strains were tested for their potentiality to produce lovastatin , a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), which responsible for the rate –limiting

step for cholesterol biosynthesis . The fungal strains were cultivated in two stage submerged fermentation followed by screening by TLC . All positive results were evaluated by confirmatory HPLC . *Aspergillus terreus* was the best lovastatin producing strain with a level of 55 mg lovastatin per liter of screening production medium , (Siamak M. Samiee , *et al* , 2003) .

Addition of sodium nitrate during solid state fermentation of *M. purpureus* CCRC 31615 improved the productivity of monacolin K and ɣ – aminobutyric acid (GABA) to 378 mg/kg and 1267.6 mg/kg , respectively . GABA productivity increased further to 1493.6 mg/kg when dipotassium hydrophosphate was added to the medium, (Yuan-Chi Su , *et al* , 2003) .

Media containing maltose, glucose and fructose as the sole carbon source yielded red pigments . Yeast extract was a superior nitrogen source for production of red pigments . The color of pigment depended greatly on the amino acid or protein to which the pigment was associated . Water solubilized pigment complexes were considerably stable to pH changes but did not with stand high temperatures , (Broder and Kochler , 1980) .

Traditionally , *Monascus* species were grown on rice by solid-state culture . For large- scale cultivation , solid – state cultures were associated with some problems such as contamination and scale – up , (Wu; W.T., *et al* , 2000) .

Monascus is a toxigenic genus of the order Eurotiales . Citrinin was investigated in type cultures of *Monascus* species by HPLC to test citrinin producing ability in the genus, eight species of *Monascus* were found to produce citrinin in YES medium in quantities varing from 65 to 480 mg/L. The results also revealed that the production of citrinin is independent of pigment production by *Monascus* species . It is suggested that safety measures should be taken when the pigments or other products from *Monascus* are utilized , (You-Zhi Wang , *et al* , 2005).

The toxicity of citrinin to humans , thus lowering the acceptability of red mold rice to the general public . When 0.5 % ethanol was added to the culture medium , the production of citrinin decreased from 813 ppb to 561 ppb while monacolin K increased from 136 mg/kg to 383 mg/kg and GABA increased from 1060 mg/kg to 7453 mg/kg . When 500 g rice was used as a solid substrate with 120 ml water and 0.3 % ethanol , the production of monacolin K at 30 ° C increased from 136 mg/kg to 530 mg/kg , GABA production increased from 1060 mg/kg to 5004 mg/kg and citrinin decreased from 813 ppb to 480 ppb , (Jyh-Jye Wang , *et al* , 2003) .

MATERIALS AND METHODS

1. Media :

Media for cultivation such as potato dextrose agar (PDA) , potato dextrose broth (PDB) , were purchased from DIFCO Laboratories Michigan , USA . Glucose was obtained from (ACROS ORGANICS) , peptone from LAB M idgplc. Com, glycerin from (EL NASR PHARMACEUTICAL CHEMICALS Co. Egypt) . Mg $S0_4.7H_20$ from Fluka –Garantle . KNO_3 from WINLAB (U.K.) and rice was obtained from local supermarket . Acetonitrile was of HPLC

purity (Merck) . All other reagents used were of the highest grade available unless otherwise indicated.

2. Strain and culture conditions :

In our study we purchased *Monascus purpureus* 1379 DSMZ , from Deutsch Sammlung Vor Microorganismus and Zellkulturen , as a lyophilized powder in a glass ampoule sealed under vacuum , was first cultured on potato dextrose agar (PDA) containing agar 15 g /L diced potatoes 300 g / L , glucose 20 g / L , to induce spore formation . After cultivation at 30 ° C for 7 days , colonies of spores that appeared on the plates were transferred one cm^2 and inoculated into 100 ml of (PDB), and incubated at 30 ° C for 4 days with shaking at 150 rpm. After incubation , 1.25 ml (5 % v/v) of the above broth was inoculated into 25 ml of the culture medium in 250 ml flasks. The culture medium was composed of rice power 30 g /L , peptone 9 g/L , glycerin 30 ml/L , glucose 110 g /L, Mg $S0_4.7\ H_2O$ 1 g / L, KNO_3 2 g/L and water to 1 liter in a 1 L flaske. The culture was incubated at 25 °C, pH 5.0 with shaking at 150 rpm for 5 days , 10 days , 14 days , 21 days and 28 days.

The influence of different carbon sources at 4 % concentration on lovastatin production was studied . The initial medium was modified from (Tzann F.Lin and Arlond L.Demain, 1991) and contained : glucose 40 g /L, NH_4NO_3 3 g / L, KH_2PO_4 6.0 g / L, K_2HPO_4 6.0 g/L, Mg $SO_4.7\ H_2O$ 0.5 g /L, KCl 0.5 g /L, Fe $SO_4.7H_2O$ 0.01 g/L, Zn $SO_4.7\ H_2O$ 0.01 g/L, $MnSO_4$. H2O 0.003 g /L, pH 6.3.

Based on our results , we devised a medium with the following changes per liter : 50 g maltose instead of 40 g glucose and for all other sugars such as mannose, fructose and dextrin we use 50 g/L ; 6.3 anhydrous MSG (monosodium glutamate) instead of 3 g NH_4NO_3 ; 1.2 g/L of KH_2PO_4 instead of 6 g/L ; 1.2 g/L of K_2HPO_4 instead of 6.0 g/L; 4 g/L of $MgSO_4.7\ H_2O$ instead of 0.5 g/L ; 0.5 g/L of KCl ; 0.01 g/L of $FeSO_4.7H_2O$; 0.01 g/L of $ZnSO_4.7H_2O$ and 0.003 g/L of $MnSO_4.H_2O$. The initial pH of the medium was changed to pH 5.5 .

3- Fermentation condition:

Monascuc purpureus 1379 was first cultured on potato dextrose agar (PDA) containing agar (1.5%) , diced potatoes (30%), glucose (2%), to induce spore formation. After cultivation at 30° C for
7 days, colonies of spores that appeared on the plates were transferred (one cm^2) and inoculated into100 ml of (PDB) and incubated at 30 °C for 4 days with shaking at 200 rpm. After incubation 1.25 ml (5%V/V) of the above broth was inoculated into 25 ml of culture medium composed of glucose 30 g/L, peptone 25 g /L, $NaNO_3$ 2 g / L, Mg $SO_4.7\ H_2O$ 1 g/L, KH_2PO_4 1 g/L, glycerol 70 ml/L ,PH 5.0 with shaking at 200 rpm for 7 days. Then added to 200 g of the sterilized rice containing mannose 4g, monosodium glutamate 2.5 g, yeast extract 2 g ,water 60 ml, PH 5.0.The mixture was incubated at 30 °C for 72 hours. Then transferred at 25 °C for 10 days with the environmental humidity being around 65% , different times of incubation were studied.

Extraction from the sample:

Monascus red rice was ground to powder (80 mesh) and 0.5 g powders was extracted with 25 ml 67% ethanol. Extraction was performed at 50 ° C for 2 h. (Siamak M.Samiee, *et al.*(2003) and Yaw.Nan Chang, *et al.*(2002).

Calculations:

Red yeast rice (Lovastatin) = At/As x Cs/Ct x2.4

Where:

At :peak response of R.Y.R (lovastatin) in chromatogram of the test solution.

As: peak response of R.Y.R. (lovastatin) in chromatogram of the standard solution.

Ct: concentration of R.Y.R (lovastatin) in the final test solution.

Cs : concentration of R.Y.R (lovastatin) in the standard solution.

The concentration of the citrinin of samples were calculated by the equation:

Cs =Cp x As/Ap

You-Zhi Wang, *et al.* (2005)

Where:

Cp : is the concentration of pure citrinin solution.

Cs: is the concentration of sample solution.

Ap : is the area of peaks of pure citrinin solution.

As : is the area of peaks of sample solution.

High performance liquid chromatography (HPLC):

Apparatus : Shimadzu , LC – 10 AV class VP

Column : STPODS – 2 (4.6 x10 mm) or equivalent.

Solvent :Ethanol 65%

Mobile phase : Acetonitrile: 0.05 M KH_2PO_4 , pH 3.5

Flow rate : 1.5 ml/min

Injected volume :20 ul

Detector :UV at 273 nm

Temprature :40 °C

RESULTS AND DISCUSSION

The influence of different carbon sources at 5% concentration on lovastatin and citrinin production were detected in each medium. Table 1 and fig. 7,8,9,10,11,12,13 showed that 0.5448 mg/600 mg lovastatin (0.0908%) was produced in case of mannose as a sole carbon source while 0.0806 mg /600 mg lovastatin (0.0134%) was produced in case of maltose but other sugars did not help in producing lovastatin in the medium as a carbon sources for *Monascus purpureus* 1379. Yaw-Nan Chang *et.al* . , (2002) found that the maximal lovastatin yield 131 mg/L average of 3 repeats appeared at the region where the respective concentrations of rice powder , peptone , glycerin and glucose were around 34.4 g/L , 10.8 g/L , 26.4 g/L and 129.2 g/L, respectively .

Citrinin production was not detected in all cultures used in this study. You – Zhi Wang , *et al.* (2005) noticed that some commercial strains contained no citrinin or only in a very low level . They found that the

production of citrinin is independent of pigment production by *Monascus* species. They suggested that safety measures should be taken when the pigments or other products from Monascus are utilized.While Jyh-Jye Wang , *et al.*,(2003) found that when 0.3% ethanol was added to 500 g rice in a solid substrate with 120 ml water the production of citrinin decreased from 813 ppb to 480 ppb.

Table (1) : Effect of different carbon sources on lovastatin and citrinin production in R.Y.R. by *Monascus purpureus*

Items	No. of Fig.	Lovastatin (mg/600mg)	Citrinin (ng)
Concentration of lovastatin	Fig (7)	0.24	Fig (13) 10 ng
Carbon source : Glucose	Fig (8)	*	*
Dextrin	Fig (9)	*	*
Mannose	Fig (10)	0.5448 (0.0908%)	*
Fructose	Fig (11)	*	*
Maltose	Fig (12)	0.0806 (0.0134%)	*

* under the detection limit

Results in table (2) and Fig 1 , 2 , 3 , 4 , 5 , 6 and 13 showed that incubation at 25 ° C for 10 days and 65 % humidity has the best result for lovastatin production 1.419 mg /600 mg (0.2365 %) while it decrease after 14 , 21 and 28 days to produce 0.31801 , 0.26173 and 0.0438477 mg/600 mg respectively . Citrinin did not produce all the period from 5 to 28 days .

Table (2): Effect of different time on fermentation of R.Y.R. for lovastatin and citrinin production by *Monascus purpureus* 1379

Time	No. of Fig	Lovastatin mg/600mg	Citrinin (ng)
Standard	Fig (1)	2.4mg /600mg	Fig (13) 10 ng
5 days	Fig (2)	*	*
10 days	Fig (3)	1.419	*
14 days	Fig (4)	0.31801	*
21 days	Fig (5)	0.26173	*
28 days	Fig (6)	0.0438477	*

*under the detection limit

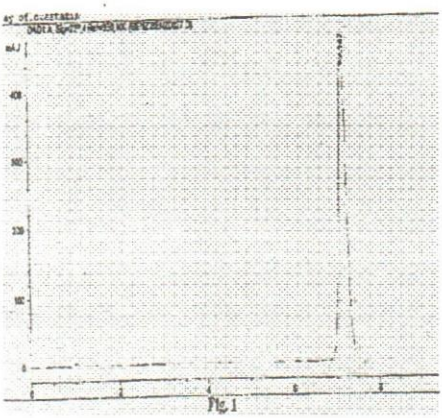


Fig. 1

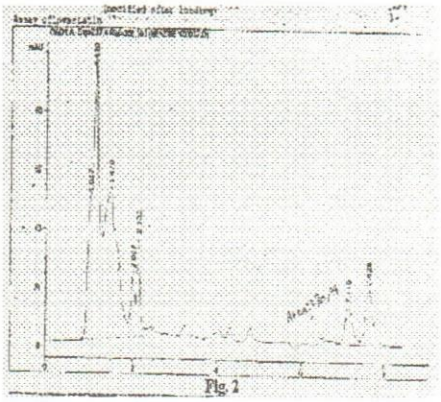


Fig. 2

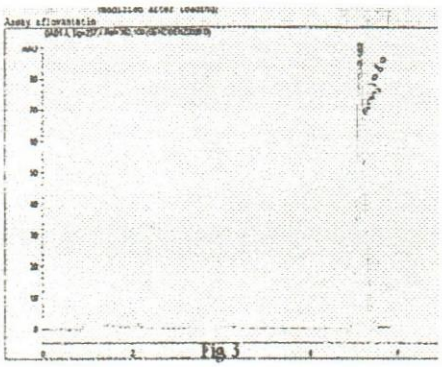


Fig. 3

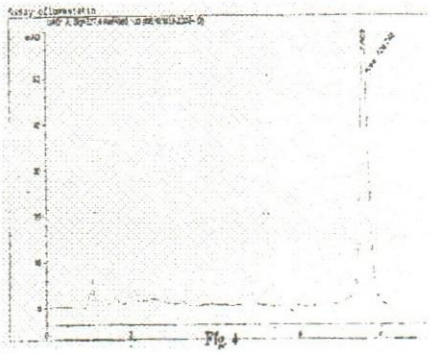
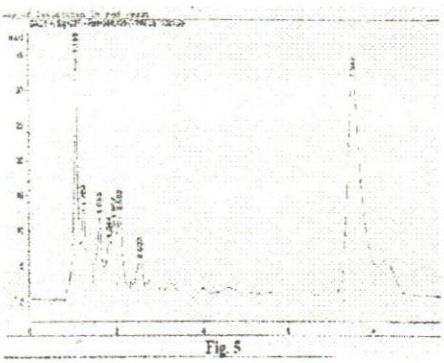


Fig. 4



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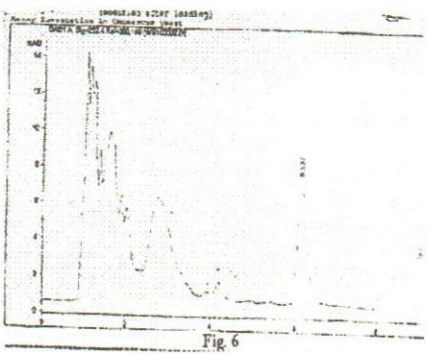

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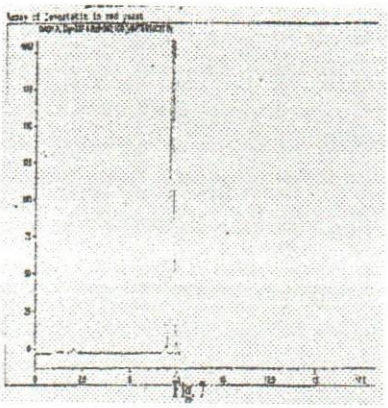

Fig.7

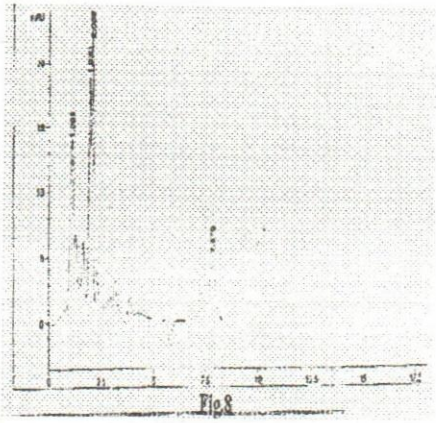

Fig.8

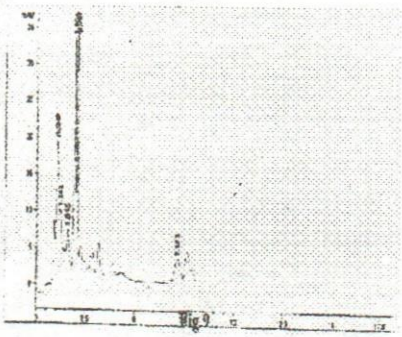

Fig.9

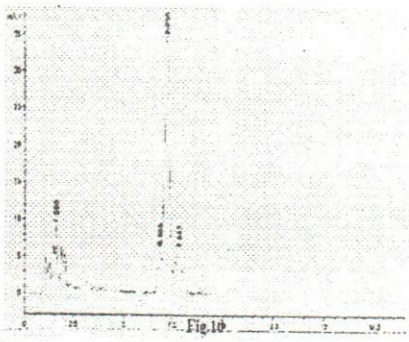

Fig.10

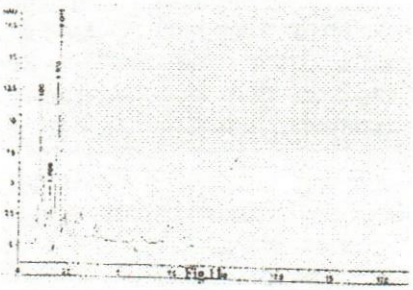

Fig.11

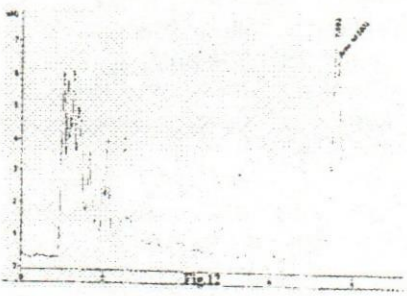

Fig.12

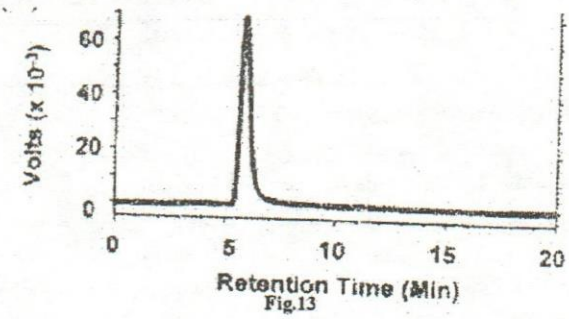

Fig.13

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إنتاج مخمر الأرز بالخميرة الحمراء بواسطة فطر *Monascus Purpureus*

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قسم الميكروبيولوجي – الهيئة القومية للرقابة الدوائية – جيزة – مصر

استعملت بيئات غذائية مختلفة لدراسة انتاج اللوفاستاتين فى مخمر الأرز بالخميرة الحمراء *Monascus Purpureus* حيث باستعمال مخلوط من البيئة الصلبة والبيئة السائلة للفطر أضيف ٢٥ مل من البيئة السائلة لكل ٢٠٠ جم من الأرز المصرى المعقم و ٤ جم من المانوز و ٢,٥ جم من أحادى جلوتامات الصوديوم و ٢ جم من مستخلص الخميرة عند درجة حموضة خمسة وتم تحضينها على درجة حرارة ٣٠ °م لمدة ٧٢ ساعة ثم نقلت لتحضن على درجة ٢٥°م فى مكان مظلم لمدة ١٠ ايام وذلك فى ظروف رطوبتها ٦٥ ٪ .

وقد وجد انه باستعمال ٢٠٠ جم من الأرز المصرى المحلى امكن الحصول على ١١٣ جم بودرة خام من مخمر الأرز بالخميرة الحمراء والذى يحتوى علي اللوفاستاتين بنسبة ٠,٢٣٦٥ ٪ .