

Cellular Localization and Production of Lovastatin from *Monascus purpureus*

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Seenivasan, *et al.*: Studies on Biosynthesis of Lovastatin

Lovastatin is the first FDA-approved antihypercholesterolemic drug for the treatment of cardiovascular diseases. *Monascus purpureus* is one of the safest molds for the production of lovastatin that has been used in the preparation of Chinese medicine Red-yeast rice. This investigation dealt with the effect of both static and dynamic culture conditions on the morphological change and localization of lovastatin in *Monascus purpureus*. In dynamic culture condition, pellet morphology was observed and the maximum intra- and extra-cellular components of lovastatin including both β -hydroxy acid and lactone forms were 1043.45 and 207.94 $\mu\text{g/l}$, respectively. Filamentous (mat) form of morphology was observed in the static culture condition and the intra- and extracellular concentration of lovastatin were 677.9 and 789.2 $\mu\text{g/l}$, respectively. Taguchi's L_{12} (11^2) orthogonal arrays was employed to find the optimal conditions for the submerged production of lovastatin and for the growth of *Monascus purpureus*. Three physical and five chemical variables were considered in the current experimental study. The maximum production of lovastatin was observed to be 3.66 mg/l. Among the chemical parameters, MnSO_4 and MgSO_4 were the most significant parameters for the production of lovastatin. Physical parameters, *viz.*, agitation rate and temperature, were also equally significant for the production of lovastatin. Ammonium chloride was the most significant parameter among the variables studied for the growth of *Monascus purpureus*, followed by glucose and phosphorous sources (KH_2PO_4). Statistical analysis showed that those parameters were significant with more than 99 % confidence ($p < 0.01$).

Key words: HMG-CoA reductase inhibitor, Static and dynamic culture conditions, pellet and mycelial morphology, optimization

Hypercholesterolemia is the presence of high cholesterol level in the blood, which is commonly associated with coronary heart disease and other complications. Statins are currently prescribed as hypocholesteremic drugs that reduce and control the blood cholesterol levels. Among statins, lovastatin ($\text{C}_{24}\text{H}_{36}\text{O}_5$, mevinolin, monacolin K) was the first FDA-approved and commercialized antihypercholesterolemic drug^[1]. It is a competitive inhibitor of β -hydroxy β -methylglutaryl-CoA (HMG-CoA) reductase, the enzyme that catalyses the rate limiting reaction of *de novo* pathway of cholesterol biosynthesis, which is responsible for producing two-thirds of the body's daily requirement of cholesterol^[1-5]. Lovastatin has served as a precursor for the next generation drugs, such as, wuxistatin and simvastatin and offered pleiotropic clinical applications^[1,6-9].

Several microorganisms, *viz.*, *Aspergillus* sp., *Monascus* sp., *Pleurotus* sp., *Phoma* sp., *Doratomyces nanus*, and *Gymnoascus umbrinus* were found to produce lovastatin through fermentation route^[1,10]. Among the organism, *Monascus* sp. has received more attention for the production of lovastatin due to its low production of cytotoxins (e.g. citrinin, a neurotoxins)^[11,12]. *Monascus* sp. (*M. purpureus* and *M. ruber*) has been employed in the preparation of Red-yeast rice (RYR), a traditional fermented food consumed in Asian countries as "red *koji*". Later, It was identified that lovastatin is

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a functional constituent of RYR^[1,13-15]. *Monascus* sp. was also exploited for various metabolites, viz., γ -aminobutyric acid, pigments, lipids, and structural analogues of lovastatin (pravastatin, compactin, monacolin J)^[13,16-19]. Endo^[20] has first reported the production of lovastatin from *Monascus* sp. in 1979. Several studies has been reported on the optimization of lovastatin production from *Monascus* sp.^[13,21-24]. In 2002, Chang *et al.*^[21] proposed a complex medium for *M. ruber*. The production of lovastatin was also carried out in solid-state fermentation by Panda *et al.*^[25] using a mixed culture of *Monascus* sp. Recent attempt on *Monascus* sp. showed that the possibility of the production of lovastatin without citrinin^[26,27]. Manzoni *et al.*^[18] have showed the production of pravastatin along with lovastatin in *Monascus* sp.

Lovastatin has two forms, hydroxy acid and lactone forms (fig. 1). However, these individual forms

have different physico-chemical and pharmaceutical properties^[30]. It was stated that hydroxy acid form of lovastatin was sparingly soluble in water than lactone form and quite stable in the liquid medium^[28]. Yang and Hwang^[29] studied the interconversion between hydroxy and lactone forms of lovastatin and reported the possible accumulation of undesired forms, such as, ester forms of lovastatin during the interconversion. Hence, the simultaneous determination of individual forms could result in the more accurate quantification of lovastatin that helps in the localization of lovastatin in the cell during the fermentation. Localization studies may help to select an appropriate downstream process as well as useful to perform an extractive fermentation and a tailor-made synthesis of lovastatin analogues, i.e., simvastatin^[7]. To the best of our knowledge, it is the first attempt to detail the localization of lovastatin content in *M. purpureus* grown under both static (non-agitated) and dynamic culture (agitated) conditions.

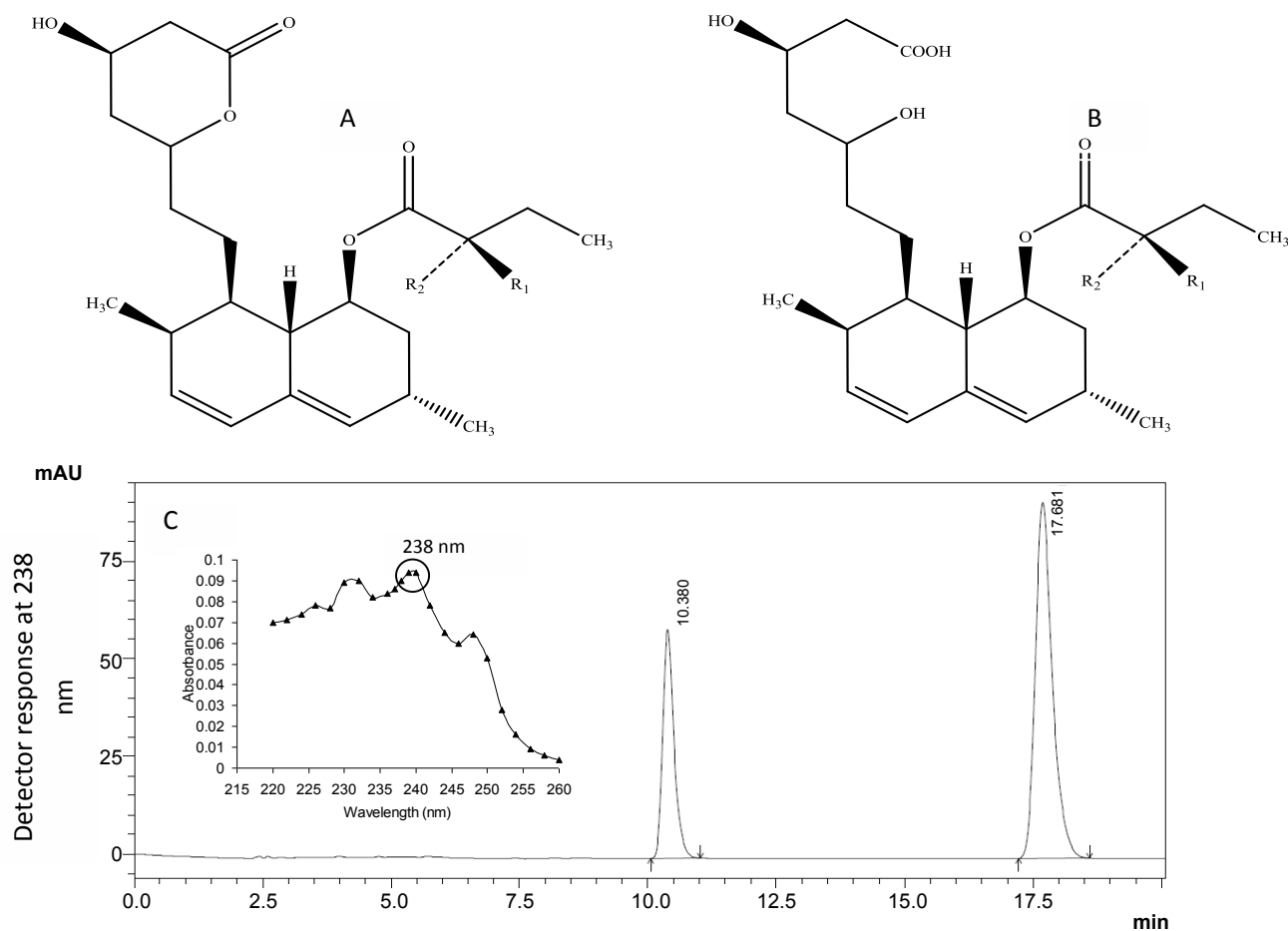


Fig. 1: Proactive lactone (closed ring) (A), active hydroxy acid (open ring) forms of lovastatin (B) and HPLC-PDA chromatogram of hydroxy acid and lactone forms of lovastatin (C)

R_1 and R_2 are the functional groups present on butyric side chain and the functional groups of R_1 and R_2 are $-OH$ and $-CH_3$ for lovastatin, respectively (A, B). Conditions employed: isocratic elution with acetonitrile and 0.1 % phosphoric acid (60:40, v/v), flow rate at 1 ml/min and column temperature 40° , injection volume is 20 μ l. The retention time of hydroxy form and lactone ring structure are 10.38 min and 17.68 min, respectively. The inner diagram showed the maximum absorbance (λ_{max}) of lovastatin at 238 nm read in spectrophotometer for the range between 220 nm and 260 nm (C)

Suitable statistical and/or non-statistical tools were employed to design the experiments and analysis the data for the optimization experiments^[30-32]. The conventional method of studying one variable at a time was very inefficient in many cases, costly, and labour intensive^[31-33]. Statistical methods were famous in the field of optimization of fermentation processes^[31]. Among statistical experimental designs, Taguchi's method has been widely used in industrial process design, principally in the developmental trials. This technique could generate enough process information to establish screening and optimal condition with a minimum number of experiments^[34]. To nullify the impact of uncontrolled (noise) factors, Taguchi has developed an orthogonal array technique, a robust and well-established engineering technique, to improve the quality or quantity of product by making it robust/insensitive to noise factors^[33,35]. As a process using a single trial with a few experiments, Taguchi's method was used successfully to optimize the production of many fermentation-derived products, viz., sucrose monoester, griseofluvin, ethanol and biomass^[36-39]. Another advantage of this method is to study the effect of both physical and chemical parameters on the desired product in a single experimental design.

The objective of the present study was to determine the localization of individual forms of lovastatin in *M. purpureus* grown in both static and dynamic culture conditions separately and to determine the optimal conditions for the production of lovastatin and biomass of *Monascus purpureus* using Taguchi's orthogonal array method.

MATERIALS AND METHODS

Mevinolin (lovastatin) was procured from Sigma Aldrich, St. Louis, MO, USA. All other chemicals were of analytical grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. The preparation of individual forms (hydroxy- and lactone forms) of lovastatin and standard chart preparation were described in detail in our previous paper^[40]. *M. purpureus* MTCC 369 was obtained from the Institute of Microbial Technology, Chandigarh, India. The organism was maintained on potato-dextrose-agar slants^[13]. The organism was sub-cultured for every 30 d of time interval. The slant cultures were grown at 30±2° and stored at 4±1° until further use.

Culture conditions:

The seed medium for the growth of the organism was described by Su *et al.*^[13] and has the composition (g/l):

glucose- 100; peptone- 10; KNO₃- 2; NH₄(H₂PO₄)- 2; MgSO₄·7H₂O- 0.5; CaCl₂- 0.2. The initial pH of the seed medium was adjusted to 6 using 2 M NaOH. One hundred millilitres of sterile seed medium contained in a 500-ml Erlenmeyer flask was inoculated with 1 ml of spore suspension containing 10⁵ to 10⁶ (spores) and incubated in a temperature controlled rotary shaker maintained at 140 rev/min and 30±1° for a period of 48 h.

The production medium had the following composition (g/l): dextrose- 29.59; ammonium chloride (NH₄Cl)- 3.86; potassium dihydrogen orthophosphate (KH₂PO₄)- 1.73; MgSO₄·7H₂O- 0.86; MnSO₄·H₂O- 0.19; pH 6^[22]. A 500-ml Erlenmeyer flask containing 100 ml of media was inoculated with 10 % (v/v) seed culture (dry weight of cell was 2±0.2 g/l) and incubated at 30° for 14 d.

Extraction of lovastatin:

About 5 ml of culture sample was harvested from 14 d old fermentation broth. Samples were homogenized by ultrasonication at 20 kHz for 20 min having pulse rate of 3 s. The homogenized fermentation broths were extracted with an equal volume of ethyl acetate at 60° for 30 min with intermittent shaking^[40]. The upper organic layers were separated from the aqueous layers by centrifugation at 4000 rpm for 20 min to settle the debris of cells. The clear organic phases (ethyl acetate) were dried under vacuum conditions in a rotary evaporator at 50°. The resultant residues were further dried under vacuum for an hour to remove the residual moisture and solvents. Finally, the dried residues were re-suspended in pure acetonitrile. This suspension was filtered through a 0.45 µm filter paper. The filtered samples were analysed by high-performance liquid chromatography (HPLC).

Quantification of lovastatin and glucose:

An analytical HPLC system was employed on a gradient mode (Shimadzu, Prominence HPLC, Kyoto, Japan), equipped with a photodiode array detector (PDA) and a Luna C18 column (Phenomenex®). The mobile phase composed of acetonitrile and acidified water (0.1 % H₃PO₄; 60:40, v/v) was filtered through a 0.22-µm membrane filter and degassed in an ultrasonic bath. The flow rate and column temperature were maintained at 1 ml/min and 40°, respectively. The sample injection volume was 20 µl. The working standards of lactone and hydroxy acid forms of both lovastatin were analysed by HPLC-PDA at 238 nm (fig. 1)^[40].

Glucose was assayed using refractive index detector

(RID) in HPLC. The aminex HPX-87H column (300×7.8 mm, i.d; Bio-Rad®) was used with the mobile phase composition of 5 mM H₂SO₄ and the oven temperature and flow rate were maintained at 50° and 0.5 ml/min. The cell free broth was centrifuged to settle the suspended particle followed by filtration with 0.45 µm nylon membrane to analyses the residual concentration of glucose along the fermentation period.

Estimation of biomass:

For the estimation of dry cell weight, a 5 ml aliquot of fermentation broth was collected from 14 d old fermentation broths and centrifuged aseptically at 4000 rpm for 30 min to separate the cell mass. The residue containing the cell mass was washed repeatedly with distilled water, followed by centrifugation until complete washout of residual medium. Later, it was dried in a hot air oven at 70° for 12 h. Before measuring the dry weight of cell, it was brought to room temperature in vacuum desiccators. The effectiveness of the drying was confirmed by measuring the dry weight at a constant interval of time until it showed concordant values.

Effect of culture condition on localization studies:

To study the effect of culture condition on the localization of lovastatin, the production medium was prepared and separately cultured in both static (without agitation) and in dynamic (agitation) culture conditions. Static cultures were incubated at 30° for 14 d on a rotary shaker without shaking and an equivalent set of liquid cultures were maintained at aforementioned condition on a rotary shaker at 120 rpm.

For localization studies, an aliquot of 10 ml of the fermentation broth was collected aseptically and centrifuged at 5000 rpm for 20 min to separate cells from fermentation broth. The clear supernatant containing extracellular lovastatin was extracted with equal volume of ethyl acetate. Cells were suspended in saline and centrifuged twice to remove the medium residues. The cell suspension was sonicated in ethyl acetate as the extracting solvent in the ratio of 1:5 (w/v) to extract the intracellular lovastatin. The condition of extraction and quantification has been discussed in the previous paragraphs.

Taguchi's orthogonal array:

Taguchi postulates two parameters as input of any process; one is measurable (controlled) factor and simple to control; another one is noise factors (uncontrolled factors), which is difficult to control. Taguchi has

developed a technique, called cross orthogonal arrays, where an orthogonal array of control variables (inner array) is crossed with an orthogonal array of noise variables (in outer array). For each setting of controlled variables, there will be “n” runs in the noise variables from the outer array^[35]. In the orthogonal array, each levels of a control variable are appears equals number of times in each columns, which helps to represent the relative effect of a level of the controlled variables over other levels and from the effect of other variables. This orthogonal array also reduces the experimental runs without compromising the pair wise balancing property^[32,33,41]. More importantly, the findings are robust and are insensitive to variations, such as, variation between batches and unknown variations.

Taguchi has also introduced the term called signal-to-noise ratio (performance criteria or loss function) that account the mean and variation in the process. There are three ways to represent the signal to noise ratio as follows: the larger, the better: $(S/N)_i = -10\log[1/n \times \sum_{i=1}^n 1/y_i^2]$; maximizing the response; the smaller, the better: $(S/N)_i = -10\log[1/n \times \sum_{i=1}^n y_i^2]$; minimizing the response; target is the best: $(S/N)_i = -10\log[s^2/\bar{y}^2]$; achieving the target value, where, $(S/N)_i$ is the signal to noise ratio of *i*th run or experiment in the design, y_i , \bar{y} , *s*, and *n* are the response of *i*th experiment, mean response, standard deviation of the response and total number of experiment..

Optimization methodology:

According to Taguchi's orthogonal array, eight variables, including five chemical parameters and three physical parameters, in twelve experiments were used to evaluate the influence of various parameters on the production of lovastatin by *M. purpureus* MTCC 369 (Table 1). Experiments were performed according to an experimental plan given in Table 1. JMP 5.1.1 statistical software (SAS Institute Inc., Cary, North Carolina, USA) was used for this investigation. The selected L₁₂(11²) orthogonal arrays (Table 1), in which, 2 levels of 8 columns (controlled factors) and 3 columns (dummy) are available within the orthogonal array.

Among the different chemical parameters studied, the following parameters such as, glucose, NH₄Cl, KH₂PO₄, magnesium sulphate (MgSO₄), and manganese sulphate (MnSO₄), have showed significant influence on the production of lovastatin^[13,22]. Hence, these parameters have been examined along with physical parameters, such as, temperature, pH and agitation speed as shown in the Table 1.

RESULTS AND DISCUSSION

There was a considerable difference in the morphology, substrate consumption, and localization of lovastatin by the organism when the culture conditions were varied between static and dynamic culture conditions. The rate of utilization of glucose was high in the

dynamic culture of *M. purpureus*. Hence, there was a rapid decrease in the pH of the fermentation medium from 6 to ~2 in dynamic culture condition within 4 d of fermentation (fig. 2). Pellets of different sizes were observed in the submerged culture condition and were highly compact at initial days of fermentation (fig. 3). The cell disintegration was observed after

TABLE 1: L₁₂ (11²) ORTHOGONAL ARRAY OF TAGUCHI'S EXPERIMENTAL DESIGN FOR PRODUCTION OF LOVASTATIN AND BIOMASS BY *M. PURPUREUS* MTCC 369

Run no.	Glucose (g/l)	NH ₄ Cl (g/l)	KH ₂ PO ₄ (g/l)	MgSO ₄ (g/l)	MnSO ₄ (g/l)	pH (Initial)	Agitation (rpm)	Temperature (°)	I	J	K	Bio-mass (g/l)	S/N ratio for biomass	Lova-statin (mg/l)	S/N ratio for lovastatin
1	1 (=5)	1 (=1)	1 (=1)	1 (=0.098)	1 (=0)	1 (=3)	1 (=110)	1 (=25)	1	1	1	6.02	15.6	1.69	4.56
2	1 (=5)	1 (=1)	1 (=1)	1 (=0.098)	1 (=0)	2 (=6)	2 (=150)	2 (=35)	2	2	2	6.36	16.07	1.14	1.14
3	1 (=5)	1 (=1)	2 (=4)	2 (=0.98)	2 (=0.447)	1 (=3)	1 (=110)	1 (=25)	2	2	2	8.27	18.35	3.56	11.03
4	1 (=5)	2 (=5)	1 (=1)	2 (=0.98)	2 (=0.447)	1 (=3)	2 (=150)	2 (=35)	1	1	2	11.04	20.86	0.24	-12.4
5	1 (=5)	2 (=5)	2 (=4)	1 (=0.098)	2 (=0.447)	2 (=6)	1 (=110)	2 (=35)	1	2	1	12.46	21.91	1.58	3.97
6	1 (=5)	2 (=5)	2 (=4)	2 (=0.98)	1 (=0)	2 (=6)	2 (=150)	1 (=25)	2	1	1	18.78	25.47	0.19	-14.43
7	2 (=40)	1 (=1)	2 (=4)	2 (=0.98)	1 (=0)	1 (=3)	2 (=150)	2 (=35)	1	2	1	15.16	23.61	0.26	-11.7
8	2 (=40)	1 (=1)	2 (=4)	1 (=0.098)	2 (=0.447)	2 (=6)	2 (=150)	1 (=25)	1	1	2	15.18	23.63	3.66	11.26
9	2 (=40)	1 (=1)	1 (=1)	2 (=0.98)	2 (=0.447)	2 (=6)	1 (=110)	2 (=35)	2	1	1	4.98	13.95	1.48	3.4
10	2 (=40)	2 (=5)	2 (=4)	1 (=0.098)	1 (=0)	1 (=3)	1 (=110)	2 (=35)	2	1	2	20.95	26.42	2.31	7.27
11	2 (=40)	2 (=5)	1 (=1)	2 (=0.98)	1 (=0)	2 (=6)	1 (=110)	1 (=25)	1	2	2	19.4	25.76	1.3	2.28
12	2 (=40)	2 (=5)	1 (=1)	1 (=0.098)	2 (=0.447)	1 (=3)	2 (=150)	1 (=25)	2	2	1	16.28	24.23	1.48	3.4

Taguchi's experimental design included five components of medium constituent and three physiochemical parameters as factors with their corresponding production levels of lovastatin and biomass by *M. purpureus* MTCC 369

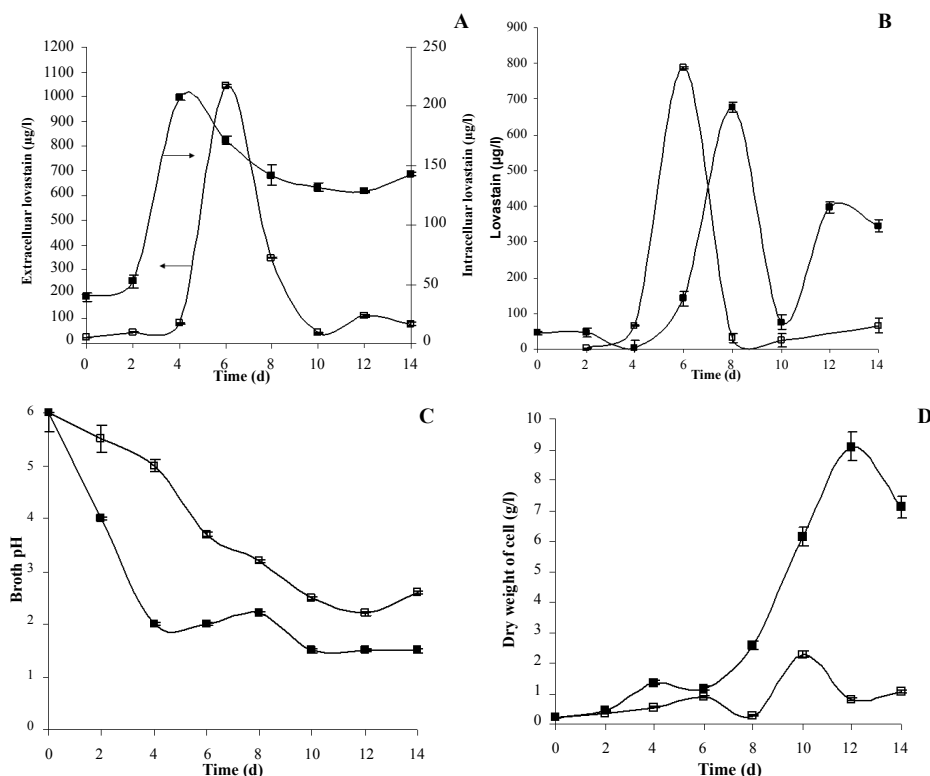


Fig. 2: The effect of culture conditions on the localization of lovastatin, and profiles of cell mass and pH of fermentation broth
Localization of lovastatin in *Monascus purpureus* in both dynamic (A) and static culture (B) conditions. (C) The pH profile of the *M. purpureus* fermented broth and dry weight of the cell (D). The ligand (-□-) and (-■-) indicates the extracellular lovastatin and intracellular lovastatin, respectively (A and B). The ligand (-□-) and (-■-) indicates static and dynamic culture conditions, respectively (C and D)

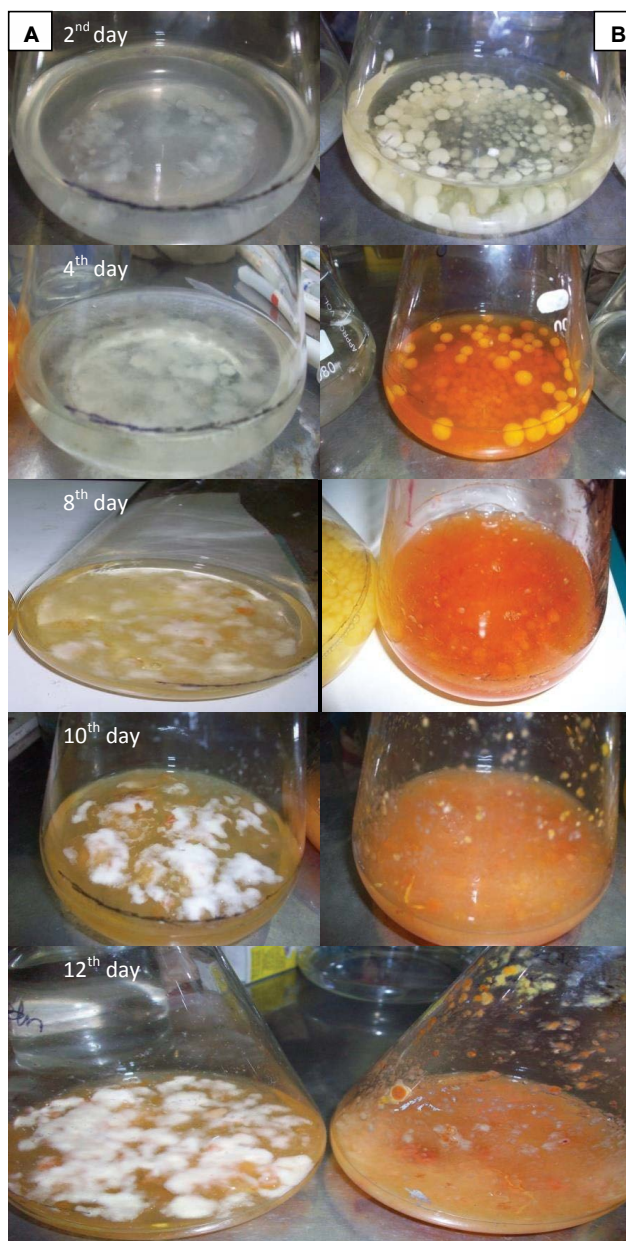


Fig. 3: Morphological observation of *Monascus purpureus* grew in both surface (A) and submerged (B) fermentations
The pelleted and filamentous growth was observed in submerged and surface fermentation, respectively along the fermentation period of 14 d

8th d of fermentation. The maximum dry weight of the cell was observed as 9.08 g/l in the dynamic culture condition (fig. 2). The apparent specific growth rate was 0.14 h⁻¹ for the initial glucose concentration of 30 g/l in the dynamic culture condition. Similarly, the specific growth rate of *Monascus* sp. was reported as 0.107 h⁻¹ at the initial glucose concentration of 20 g/l in a dynamic culture condition^[17]. Similarly, Hamdi *et al.*^[42] found the specific growth rate of *M. purpureus* to be between 0.05 and 0.1 h⁻¹. Musaalbakri *et al.*^[43] has reported the maximum specific growth rate as 0.055 h⁻¹.

In static culture condition, surface (filamentous) growth morphology was observed throughout the fermentation process with the apparent specific growth rate of 0.065 h⁻¹ and there was no cell disintegration in the static culture condition. The thickness and density of the filamentous morphology had increased along the fermentation period (fig. 3). Under the static culture conditions, the value of pH dropped gradually from 6 to ~3 over the entire fermentation period of 14 d. The maximum cell concentration of 2.28 g/l was observed in static culture (fig. 2).

There was considerable difference in the localization pattern of lovastatin in the dynamic culture condition compared to the static culture condition. The production of hydroxy acid and lactone was maximum in intracellular (5 fold higher) and extracellular (19 fold higher) compartment in the agitated or dynamic culture, respectively. From the present study, it has been observed that the interconversion between the hydroxy acid and lactone form of lovastatin was influenced by the pH of the fermentation period, which could be one of the reasons for its maximum accumulation of lactone form of lovastatin outside the cell than its hydroxy acid form. Since, the hydroxy acid form was converted into lactone form and other intermediates (monacolins) when the system pH was less than three or under acidic conditions^[44,45].

In the dynamic culture condition, the maximum extracellular lovastatin content was found to be 1043.56 µg/l and the maximum intracellular content of lovastatin was 207.94 µg/l (fig. 2). The maximum extracellular localization of lovastatin could be due to easy mass transfer of lovastatin through the cell in dynamic (agitated) culture condition. The initial rate of formation of extracellular lovastatin was found to be 222.41 µg/l/d in the dynamic culture condition. The maximum yield ($Y_{p/X}$) of lovastatin was found to be 1.45 mg/g of biomass (Table 2).

Another reason for the maximum extracellular localization of lovastatin was due to the cell lysis (pellet degradation) in the final days of fermentation (fig. 3). In the meantime, cells have maintained a steady state lovastatin profile inside the cell. Cells have reached their maximum holding capacity of lovastatin (i.e. ~200 µg/l) after 4 d of fermentation (fig. 2). A slight decrease in this concentration of intracellular lovastatin could be due to the cell lysis. It was observed that lovastatin could itself restrict its own production as it is shown in *Aspergillus* sp., would be the probable

reason for its holding limits^[46]. The amount of lactone form of lovastatin was found more than that of hydroxy acid form (26 fold higher) in the extracellular portion of static culture, whereas, the localization of hydroxy acid form was more than the lactone form (6 fold higher) within the cell.

In static culture of *M. purpureus*, the maximum extracellular localization of lovastatin was observed to be 789.2 µg/l, whereas, the maximum intracellular content of lovastatin was 677.90 µg/l (fig. 2). The initial rate of the extracellular production of lovastatin was 214.2 µg/l/d (Table 2). Similarly, the initial rate of intracellular production of lovastatin was 85.7 µg/l/d. The maximum yield of lovastatin in the static culture condition was observed to be 1.39 mg/g of biomass.

In static culture condition, the concentration of intracellular localization of lovastatin was comparable to the extracellular content of lovastatin and it was high as compared to the intracellular content of lovastatin in the dynamic culture. It corroborated the significance of agitation on the localization of lovastatin in *M. purpureus*. Cells could easily find the substrate and release the product in the dynamic culture condition, whereas, in the surface culture, the mass transfer resistance limits the overall yield of lovastatin.

There was a variation in the profile of lovastatin, which could be due to the pH of the fermentation broth and medium complexity. At low pH, the conversion between the individual forms of lovastatin and its analogues were random^[44,45], which brings variation in the profile of lovastatin.

On comparing the production of lovastatin in the static and dynamic culture conditions, it could be seen that the production of total lovastatin (sum of both intra and extracellular) in both culture has not been significantly varied. However, the extracellular localization of lovastatin was high in the dynamic culture compared to static culture, which could help lovastatin to be extracted easily from broth. The extraction of lovastatin from intracellular portion of cell complicates

the downstream processing due to the presence of the structural analogues and intermediates of lovastatin^[47]. It is very difficult to separate them from lovastatin that would increase the overall production cost.

Submerged batch fermentation studies were carried out for the production of lovastatin by *Monascus purpureus* using Taguchi's design of experiment (Table 1). The experimental results showed a significant variation in the production of lovastatin (Table 3 and fig. 4A). Maximum production of lovastatin was 3.66 mg/l for the condition using the experimental run number 8 (Table 1). Minimum production of lovastatin was 0.19 mg/l obtained using condition of run 6. There is no difference in the production of lovastatin using conditions of either the run 9 or the run 12. Maximum production of lovastatin was obtained using the medium, whose composition was (g/l): glucose- 40; NH₄Cl- 1; KH₂PO₄- 4; MgSO₄- 0.098; MnSO₄- 0.447 (Table 1).

From Table 3 and main effect plot for signal-to-noise ratio (fig. 4A), it can be observed that the parameters, viz., NH₄Cl (at lower level), KH₂PO₄ (at higher level), MnSO₄ (at higher level), and MgSO₄ (at lower level) had significantly contributed to the production of lovastatin (Table 3 and fig. 4A). Glucose was relatively insignificant for the production of lovastatin compared to other parameters. The glucose is significant for the growth of cell than the production of secondary metabolites, lovastatin, may be due to the effect of catabolic repression. NH₄Cl was one of the least significant variables for the production of lovastatin at its lower level, whereas, the same have shown significant influence on the growth of *M. purpureus* at its higher level. It showed that the limitation of nitrogen has enhanced the production of secondary metabolite, lovastatin. Main effect plot for the production of lovastatin showed the influence of individual parameters (fig. 4A).

Among the physical parameters, agitation speed and

TABLE 2: LOCALIZATION OF MAXIMUM CONCENTRATION OF LOVASTATIN IN BOTH STATIC AND DYNAMIC CULTURES OF *M. PURPUREUS* WITH YIELD CO-EFFICIENTS

Condition	Fraction	Max. product conc. (µg/l)	DWC (g/l)	Initial rate (µg/l/day)	Max. Y _{X/S} (g/g)	Max. Y _{P/X} (mg/g)	Max. Y _{P/S} (mg/g)
Static culture	Extracellular	789.20 (6 th)*	2.28 (10 th)*	214.2	0.11	1.39	0.060
	Intracellular	677.90 (8 th)*		85.7			
Dynamic culture	Extracellular	1043.45 (6 th)*	9.08 (12 th)*	222.41	0.29	1.45	0.040
	Intracellular	207.94 (4 th)*		47.17			

*indicates the corresponding day for the maximum concentration of lovastatin and dry weight of the cell (DCW), where μ_{app} is the apparent specific growth rate (h⁻¹)

TABLE 3: ANALYSIS OF THE DATA FOR DETERMINATION OF INFLUENCING FACTORS ON THE PRODUCTION OF LOVASTATIN AND BIOMASS

Variable	Variable in actual units	Levels*	Sum	Mean	SS	% SST	Sum	Mean	SS	% SST
Lovastatin						Biomass				
A	Glucose, g/l	1 (=5)	8.4173	1.4029	0.357	3.03	62.937	10.4895	70.2252	20.92
		2 (=40)	10.4872	1.7479			91.9663	15.3277		
B	NH ₄ Cl, g/l	1 (=1)	11.7966	1.9661	1.8319	15.52	55.9728	9.3288	153.78	45.82
		2 (=5)	7.108	1.1847			98.9305	16.4884		
C	KH ₂ PO ₄ , g/l	1 (=1)	7.3391	1.2232	1.4886	12.61	64.077	10.6795	59.6273	17.77
		2 (=4)	11.5655	1.9276			90.8263	15.1377		
D	MgSO ₄ , g/l	1 (=0.098)	11.8636	1.9773	1.9382	16.42	77.2709	12.8785	0.0109	0.003
		2 (=0.98)	7.041	1.1735			77.6325	12.9387		
E	MnSO ₄ , g/l	1 (=0)	6.9031	1.1505	2.1661	18.35	86.6889	14.4482	28.4424	8.47
		2 (=0.447)	12.0015	2.0002			68.2144	11.3691		
F	pH initial	1 (=3)	9.5513	1.5919	0.0033	0.03	77.7463	12.9577	0.0289	0.01
		2 (=6)	9.3533	1.5589			77.1571	12.8595		
G	Shaker speed, rpm	1 (=110)	11.9299	1.9883	2.0462	17.34	72.1048	12.0175	9.5296	2.84
		2 (=150)	6.9747	1.1624			82.7985	13.7998		
H	Temperature, °C	1 (=25)	11.8844	1.9807	1.9718	16.71	83.9293	13.9882	13.9864	4.17
		2 (=35)	7.0201	1.17			70.9741	11.829		

SS- sum of squares, SST- total sum of squares. Parentheses indicate actual value of the variable

temperature were significant for the production of lovastatin at their lower level (fig. 4A and Table 3). The initial pH did not influence much on the production of lovastatin at both the levels.

Contour plots were drawn between the production of lovastatin and influencing parameters. Fig. 4B showed the combined effect of agitation rate and MnSO₄ on the production of lovastatin, where the production of lovastatin was high, when the agitation speed and MnSO₄ were at lower and higher levels, respectively. Similarly, fig. 4C showed the effect of MgSO₄ and agitation speed on the production of lovastatin; both factors are significant at their low levels. Effect of physical parameters, such as, agitation speed and temperature on the production of lovastatin has been explained in fig. 4D and were significant at their lower level. Similarly, MgSO₄ (at lower level) and MnSO₄ (at higher level) were influenced on the production of lovastatin (fig. 4E). The curvature in the contour plot indicates that there was possible interaction between the parameters otherwise it should give a straight flat surface.

At higher agitation rate, the production of lovastatin was observed to be low, which might be due to the shear sensitivity of the organism. Shear sensitiveness of *Aspergillus* sp. was reported and it drastically reduced the production of lovastatin^[48,49]. Similarly, Barrios-González *et al.*^[50] reported that *Monascus* sp. has produced high amount of lovastatin in the solid-

state fermentation than in the submerged fermentation. However, it is interesting to note that, at higher agitation speed, the production of biomass was high. Hence, the drop in the production of lovastatin at high agitation is not associated with shear sensitivity of the cells; rather it could be associated with dissolved oxygen level of the medium. Since, agitation rate was linked with the mass transfer of substrate, product and dissolved oxygen level. In the biosynthesis of lovastatin, the oxidation of monacolin L led to monacolin J, one of the intermediates in the biosynthesis pathway of lovastatin^[1,51]. More amount of dissolved oxygen might lead to high accumulation of intermediates that could reduce the production of final product, lovastatin. At high agitation rate, there could be more chances that the organism can self-regulate its production of lovastatin. Since the contact between the cell and product, lovastatin was inevitable due to the high mass transfer rate. Since, lovastatin was observed to inhibit its own synthesis as reported by López *et al.*^[46] and Jia *et al.*^[52]. These could be probable reasons for lower production of lovastatin at high agitation speed.

Moreover, the production of lovastatin was observed to be high at its lower temperature level (25°). Tsukahara *et al.*^[53] reported a 30-fold increase in the production of lovastatin without citrinin when there was a shift in the temperature from 30° to 23°. The above results showed a strong thermodynamically favourable condition towards the production of lovastatin at lower temperature.

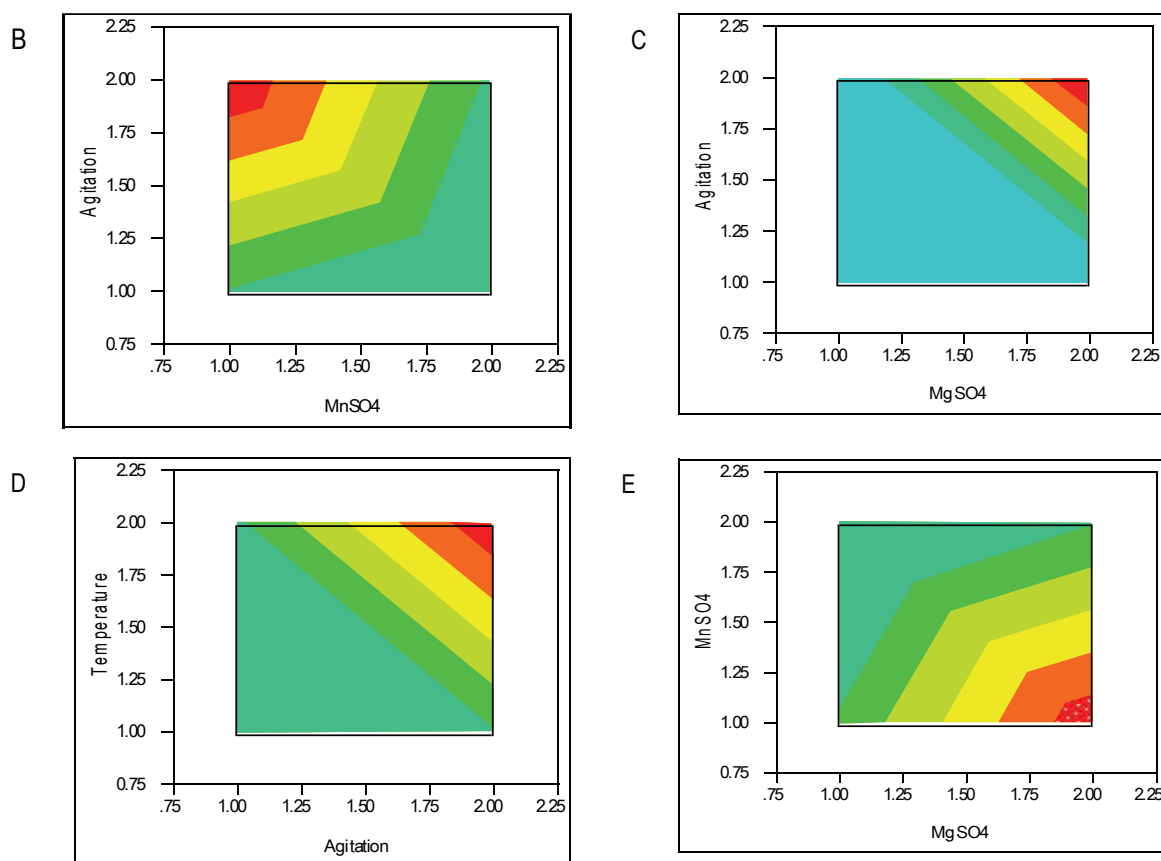
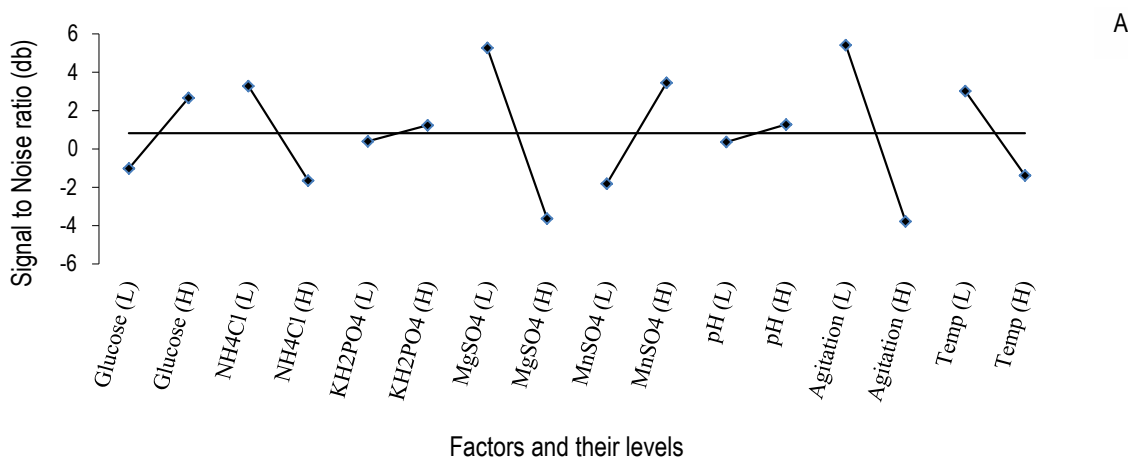


Fig. 4: The main effect plot for the production of lovastatin

Relative influence of both physical and chemical parameters on the production of lovastatin is represented at their lower (L) and higher (H) levels. Contour plots (B-E) shows the combined effect of significant parameters (MnSO_4 , MgSO_4 , temperature and agitation) on the production of lovastatin. Lovastatin (mg/l): ■ = 0.750; ■ = 1.000; ■ = 0.750; ■ = 1.500; ■ = 1.750; ■ >1.750

More quantitatively, the effect of each parameter on the production of lovastatin can be accurately estimated using the Phadke method^[32,54] as given in Table 3. Difference in the sum of production of lovastatin between low and high levels of MnSO_4 was higher

than other chemical parameters, whose contribution (i.e., total sum of square) was 18.35 %. Similarly, the difference in the sum of production of lovastatin between low and high levels of MgSO_4 and NH_4Cl were also high. The total sum of square of MgSO_4

and NH_4Cl were 16.42 and 15.52, respectively. If the difference of the sum of lovastatin production between the levels is very low, it indicates the relative influence of the parameter on the production of lovastatin is insignificant. It was observed for the case of glucose and initial pH ($\text{SST} < 3\%$). The levels of significance of both physical and chemical factors on the production of lovastatin are represented in the Pareto plot (fig. 5). It showed that among the physical and chemical parameters, MnSO_4 (at higher level) and agitation speed (at lower level) had showed the maximum contribution towards the production of lovastatin. It also showed that physical parameters were contributed significantly to the production of lovastatin as equally as that of chemical parameters, except initial pH. This showed that there are more scope and necessitates to incorporate physical parameters in the further process development of lovastatin. Similar results were observed by Dasu *et al.*^[32] for the production of griseofulvin production by *P. griseofulvum* MTCC 1898 where, they also have emphasized the contribution of physical parameters, such as, pH, agitation, and aeration on the production of griseofulvin. Those physical parameters have showed a maximum contribution on the production of griseofulvin than that of chemical parameters in batch reactor experiments.

The ANOVA analysis also showed the significance of MnSO_4 , MgSO_4 , agitation speed, and temperature on the production of lovastatin with more than 75 % confidence ($p < 0.25$). Dikshit and Tallapragada^[55] have also showed the production of lovastatin by *Monascus* sp. was highly influenced by MgSO_4 out of the screened variables. Further, the current results were close to the experimental observation of Sayyed *et al.*^[22] and have showed the interaction among the chemical parameters. Chung *et al.*^[23] has also employed the Taguchi's orthogonal array method for the finding the optimal chemical composition for the maximum production of lovastatin. They have

observed that whole wheat flour (1 %), peptone (1 %), soy bean (0.01 %), and KH_2PO_4 for the production of monacolin K.

The maximum biomass concentration of 20.95 g/l was observed for the experimental run 10, whose composition was (in g/l): glucose- 40; NH_4Cl - 5; KH_2PO_4 - 4; MgSO_4 - 0.098. The least biomass concentration was observed with experimental run 9 (Table 1). The selected physical and medium constituents showed a significant contribution to the growth of cells (Table 3) as also observed from main effect plot (fig. 6A). From the main effect plot, it could be observed that NH_4Cl (nitrogen source), glucose (carbon source), and KH_2PO_4 (phosphorous source) were the most significant parameters for the growth of the cell. In physical parameters, agitation speed and temperature are the significant parameters. Fig. 6B demonstrated the combined effect of nitrogen source and carbon source on the production of cell mass. The biomass was found to be increased with the increase in the concentration of glucose and NH_4Cl . Similar trend was also observed for the phosphorous sources. The contour plot (fig. 6C) showed the production of cell mass as a response by varying the carbon (glucose) and phosphorous (KH_2PO_4) sources. Both parameters were significant on the cell mass formation at their high level. The same trend was observed for the production of cell mass on varying phosphorous and nitrogen sources (fig. 6D). Interestingly, the production of biomass dropped at low agitation speed and high level of temperature (fig. 6E). The agitation rate was linked with numbers of pellet formation. In high-agitated system, the number of pellet formation was high, which could be probable reason for the better biomass at high agitation speed.

The relative significance of these parameters can also be found from the percentage of sum of squares values for the production of biomass (Table 3). The nitrogen (NH_4Cl) source contributed more when compared

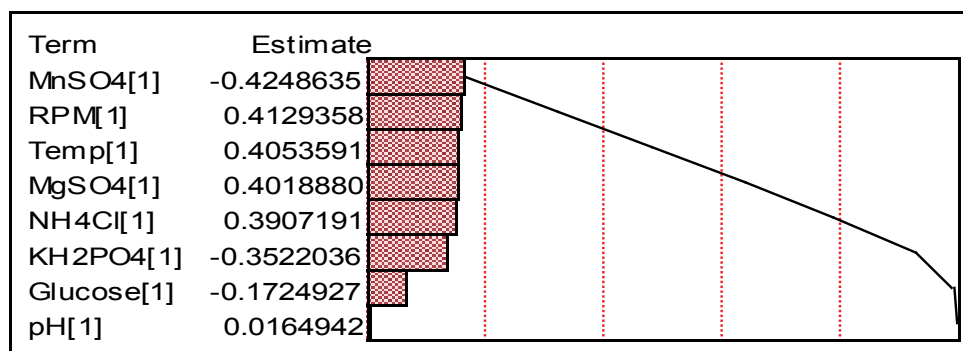


Fig. 5: Pareto chart for significant parameters for the production of lovastatin

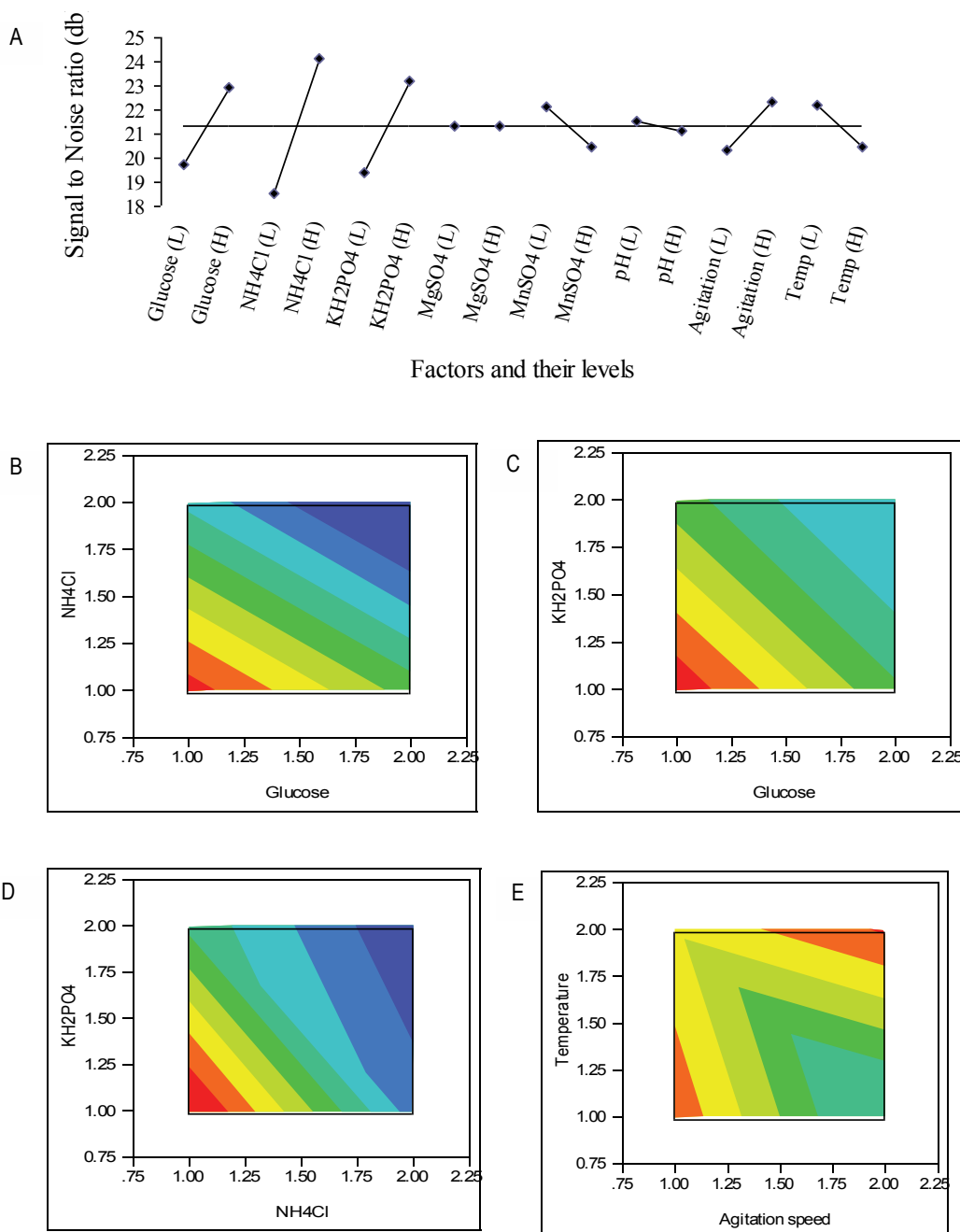


Fig. 6: The main effect plot for the growth of cell

Relative influence of both physical and chemical parameters on the production of biomass is represented at lower (L) and higher (H) levels. Contour plots (B-E) shows the combined effect of significant parameters (NH₄Cl, glucose, KH₂PO₄, temperature and agitation) on the growth of cells. Lovastatin (mg/l): ■ = 0.750/0.875/11; ■ = 0.875/10/12; ■ = 10/11.25/13; ■ = 11.25/12.5/14; ■ = 12.5/13.75/15; ■ = 13.75/15; ■ = 15; ■ = 16.25; ■ >16.25

to carbon and phosphate source to the growth of the cell. Glucose, NH₄Cl and KH₂PO₄ had the relative contribution of 20.92, 45.82, and 17.77 %, respectively (Table 3). Chung *et al.*^[23] have also revealed that KH₂PO₄ was a significant parameter for the growth of *Monascus* sp. The agitation and temperature had a relatively low significance on the cell growth. Moreover, initial pH and MgSO₄ were insignificant variables for the cell growth compared to other factors

irrespective of their levels. The Pareto diagram showed the relative significance of these parameters for the production of biomass (fig. 7). The least contributing factor is MgSO₄, whose percentage of sum of square is less than 0.003 %.

The difference in the production of biomass between the lower and higher levels of physical parameters is relatively insignificant (Table 3). Hence, compared to the physical parameters, the contribution of chemical

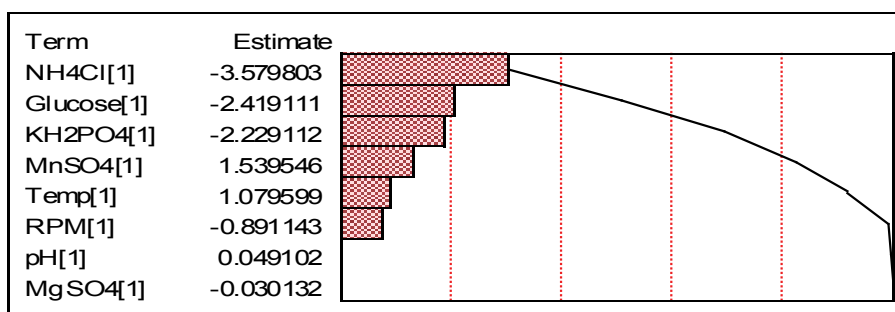


Fig. 7: Pareto chart for significant parameters for the production of cell mass

parameters was relatively more significant. It just opposite of the results obtained for the production of lovastatin. Especially, NH₄Cl is the most significant parameter in the production of biomass at its higher level, whereas, it is the least significant parameter among the significant parameters at its lower level for the production of lovastatin (Table 3 and figs. 4 and 6). In contrast, MgSO₄ is the one of the significant parameters in the production of lovastatin, whereas, the same was insignificant on the production of cell mass. It showed the strong role of MgSO₄ (at low level) in the biosynthesis pathway of lovastatin. Interestingly, parameters and their relative influences were observed inversely between the production of lovastatin and cell growth, which strongly ascertains the condition of growth limitation favoured the production of secondary metabolite, lovastatin.

Chemical parameters, such as, glucose, ammonium chloride, potassium dihydrogen phosphate, had showed a high level of significance with more than 99 % confidence ($p < 0.01$). Similarly, MnSO₄ is also another significant compound with 97 % confidence.

There was a significant variation in the morphology and localization pattern of lovastatin in *M. purpureus* when the culture condition changed. However, there was no considerable variation in the production profiles of lovastatin among the culture conditions. It has seen that the maximum localization of lovastatin was observed outside the cell in the case of dynamic or agitated culture. Hence, *M. purpureus* has to be grown on dynamic modes of fermentation to have simple downstream processing for large scale operations. The Taguchi's robust experimental design has been adopted to find the relative significance of both physical and chemical parameters. On comparison with the chemical parameters, the physical parameters, viz., agitation rate and temperature, have also been found equally significant on the production of lovastatin.

These results again corroborated the importance of physical parameters on the production of lovastatin, which needs to be given special attention in the future process development. Chemical parameters were more significant for the production of cell mass compared to physical parameters. By the Taguchi's orthogonal method, the optimal conditions for the production of lovastatin and growth of *M. purpureus* have been established. However, there was a significant interaction between the parameters as observed from the non-linearity of response in contour diagram. The current finding may be nearest to the optimal value based on the range selected.

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There are no conflicts of interest.

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