REVIEW ON PRODUCTION OF STATINS BY FERMENTATION TECHNOLOGY

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Abstract : The beneficial effects of statins are the result of their capacity to reduce cholesterol biosynthesis, mainly in the liver as well as to the modulation of lipid metabolism, procured from their result of inhibition upon HMG-CoA reductase. Statins have antiatherosclerotic effects, that positively correlate with the percent decrease in LDL cholesterol. In addition, they can exert antiatherosclerotic effects independently of their hypolipidemic action. For the reason that mevalonate metabolism generates a series of isoprenoids vital for different cellular functions, from cholesterol synthesis to the control of cell growth and differentiation, HMG-CoA reductase inhibition by statins has beneficial pleiotropic effects. The objective of the present study is summarize the production of statins by the application of fermentation technology using various filamentous fungi including *Pencillium, Monascus ruber, Aspergillus terreus, Monascus purpurea*, etc. Production of statins by fermentation decreases the production cost compared to costs of chemical synthesis.

Keywords: HMG CoA Reductase, Statins, Atherosclerosis, Fermentation

I. INTRODUCTION :

It is illustrious that raised cholesterol levels increase the risks of heart disease and stroke. Globally, a third of ischaemic heart disease is attributed due to high cholesterol and according to the World Health Organization, raised cholesterol is estimated to cause 2.6 million deaths (4.5% of total) and 29.7 million disability adjusted life years [1]. In this sense, inhibitors of 3-Hydroxy-3-Methyl Glutaryl Coenzyme A (HMG-CoA) Reductase, commonly known as statins (Figure 1), are the largest selling class of drugs prescribed for the pharmacological treatment of hypercholesterolemia and dyslipidaemia[2,3], and it has also been reported that since their introduction in 1987, the lives of millions of people have been extended through statin therapy and more importantly, quality of life has been drastically improved[4]. Since the discovery of the first statins from natural sources, mevastatin, also named compactin, from the fungi *Penicillium citrinum* [4,5] and *Penicillium brevicompactum*[6]. The economic impact of statins on the drug market is enormous. For instance, simvastatin was originally developed by Merck under the brand name Zocor™; In 2005, Zocor™ was Merck's bestselling drug and the second-largest selling statin in the world (more than US$3 million only in USA, according to different reports[7-11]. In 2006, Zocor™ went off patent, and the annual sales drastically dropped; anyhow, from that moment, generic simvastatin became the most-prescribed statin in the world between 2010 and 2015 [12-15].

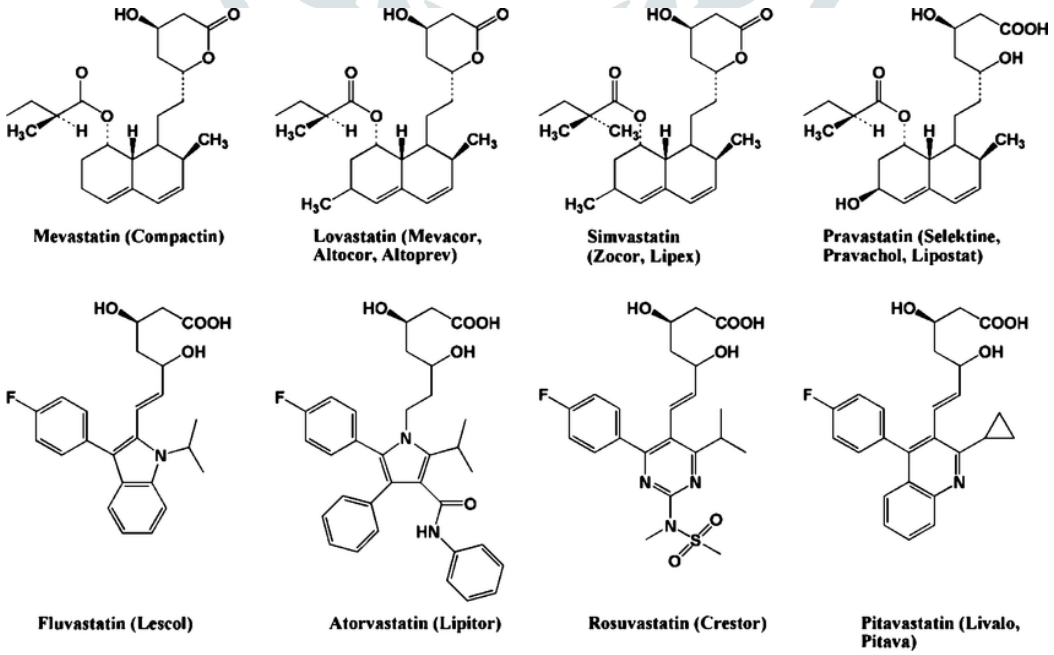

Figure 1. Some Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.

II. DISCOVERY OF STATINS:

Compactin, discovered by Akira Endo in 1973 as a structural analogue for the HMG-CoA substrate, was processed by *Penicillium citrinum*. Lovastatin, formerly known as mevinolin K and mevinolin, was produced from cultures of *Monascus ruber* and *Aspergillus terreus* respectively. It was the first statin to be approved by the FDA in 1987[16].

Pravastatin, the semi-synthetic derivative of compactin was managed in 1989. Simvastatin remains a commonly prescribed statin[17,18].

III. TYPES OF STATINS:

Statins comprise well-known medications such as atorvastatin (Lipitor), simvastatin (Zocor), lovastatin (Mevacor), pravastatin (Pravachol), rosuvastatin (Crestor) and others. Lower cost generic categories of many statin medications are available. The statins differ with respect to their ring structure and substituents. These dissimilarities in structure influence the pharmacological properties of the statins.

Sometimes, statins have been grouped into two groups of statins according to their structure. Statins that belong to type 1 are pravastatin and simvastatin. Statins that are fully synthetic and have larger groups linked to the HMG-like moiety are often referred to as type 2 statins. Statins that belong to this group are atorvastatin and rosuvastatin.

There are five statins currently used as clinical use. Lovastatin and pravastatin (mevastatin derived) are naturally statins of fungal origin while simvastatin is semi-synthetic lovastatin derivative. Atorstatin and fluvastatin are synthetic statins, which derived from mevalonate and pyridine. Lovastatin, simvastatin and pravastatin are derived from fungi. Simvastatin is chemically modified 2,2-dimethyll butyrate analogue of lovasatin. Pravastatin then is a purified active metabolite of mevastatin with an open hydroxyl acid instead of lactone ring. The first representative of the new class of statin compounds was mevastatin which is derived from a strain of *Penicillium citrinum*. Lovastatin is a natural product while simvastatin is derived from lovastatin. Pravastatin also derived from the natural products and fluvastatin is totally synthetic racemic mixture. The fungal products lovastatin, pravastatin and simvastatin are structurally related since they have a hydro naphthalene in common and differ only at a few sites in the molecule. In the present review, the production of natural statins by fermentation is summarized[19].

IV. MECHANISM OF ACTION OF STATINS:

Prominent mechanism of action is through the competitive, reversible inhibition of HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis. HMG-CoA reductase catalyses the conversion of HMG-CoA to L-mevalonate and coenzyme A via a four-electron reductive deacetylation. The pharmacophore of all statins bears resemblance to the endogenous HMG-CoA moiety , it competitively binds to the catalytic domain of HMG-CoA reductase, causing steric hindrance and preventing HMG-CoA from accessing the active site[20,21].

Through inhibition of HMG-CoA reductase, statins eventually prevent the endogenous production of cholesterol. Additionally, the resultant reduction in cholesterol concentration within hepatocytes triggers up-regulation of low-density lipoprotein (LDL)-receptor expression, which promotes the uptake of LDL and LDL-precursors from systemic circulation[22]. Consequently, a significant proportion of statins cholesterol-lowering is a result of the indirect increase in LDL clearance from plasma, as opposed to simply reduced cholesterol biosynthesis. Secondary mechanisms of statin-induced lipoprotein reduction include inhibition of hepatic synthesis of apolipoprotein B100, and the reduced synthesis and secretion of triglyceride-rich lipoproteins [23,24]

Overall, the effect on the lipid profile is consistent between statins, with reductions in total cholesterol, LDL, and triglycerides, and an increase high-density lipoprotein [25].

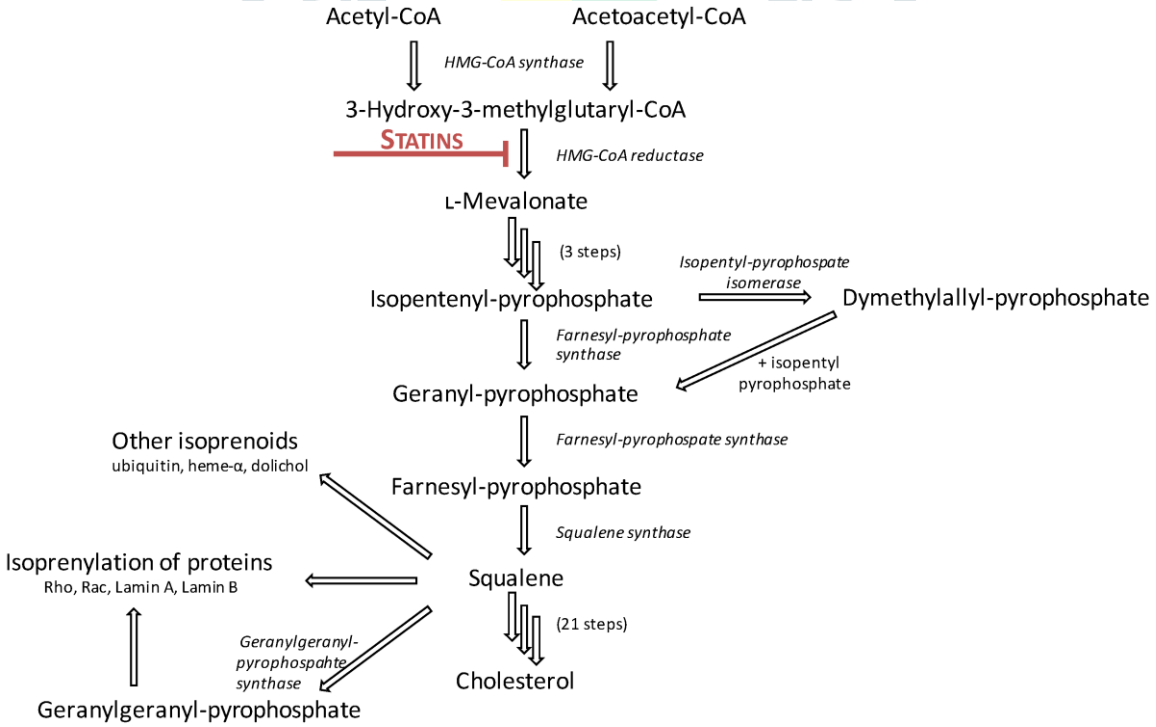

Figure 2. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to L-mevalonate, the rate-limiting step of the cholesterol synthesis pathway.

V. PRODUCTION OF STATINS:

- **Different types of microbes used in fermentation:**
  Since the discovery of the first statin from natural sources, mevastatin also named as compactin from the fungi *Penicillium citrinum* and *Penicillium brevicompactum*.Lovastatin(mevinolin,found in *Aspergillus terreus* and food such as oyster mushrooms and red yeast rice ,simvatatin (mevacor) is also isolated from *Aspergillus terreus* and pravasatin intitially known as Cs-514, originally indentified in the bacterium *Nocardia autotrophica* and is also isolated from *Penicillium chrysogenum*.

- **Physico-chemical properties**:
  Pravastatin is extremely hydrophilic and lovastatin, simvastatin, atorvastatin are hydrophobic[26].

- **Fungal fermentation and statin production**:
  Statins are manufactured as a secondary metabolite from a polyketide pathway. This pathway is regulated by polypeptide synthase genes such as Lov B, lovF and LovD, that are responsible for the transcription regulation and production of these secondary metabolites [27, 28]. Statins are produced as a secondary metabolite during stress of the fungi. Acetyl Co-A acts as precursor molecule that plays an important role in bridging the primary metabolism with the secondary metabolism leading to the production of various secondary metabolites such as terpenes and polyketides including statins[29,30] . All fungi producing lovastatin or compactin utilise the pathway. Lovastatin is commercially produced by fermentation of *A. terreus* and simvastatin is produced by further chemical treatment of lovastatin usually involving direct alkylation[31,32].

- **Production of simvastatin and lovastatin**:
  For centuries, *Monascus sp.* has been used widely in Asia as Chinese cheese, red wine and sausages. *Monascus sp*. widely used long time ago as a folk medicine for the food digestion and also blood circulation and also as a treatment of other sickness . *Monascus sp* belong to the Ascomycetes group and family of Monascaceae. *Monascus purpurea* easily can be distinguished by its ascosporic which is in spherical shape . *Monascus sp*. are nonpathogenic and use in the food processing to obtain the aroma, nutrition and also colour of the fermentation products .Using *Monascus sp* for the production of the lovastatin indicate that give advantageous with an increased saving in cost and if using directly as a functional food as long it is proves to be nontoxic . Various of active ingredients including lovastatin owned by the *Monascus purpurea* [19].

- **Lovastatin:**

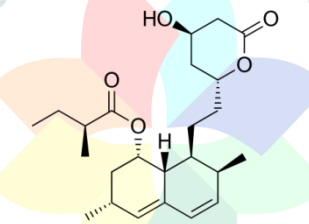

Figure 3. Lovastatin

- **Strains:** Lovastatin is a secondary metabolite produced by a variety of filamentous fungi such as *Monascus ruber, M. purpurea, M. pilosus, M. anka, Penicillium citrinum, Paecilomyces varotii*[33] etc.

- **Media:** By Solid State Fermentation Two grams of ragi bran was weighed and moisture content was maintained at 70%. One milliliter of spore suspension (108 spores) of A. terreus / *M. purpurea* was added to the sterilized substrate and incubated at 28ºC.
  After eight days of incubation, the solid substrate along with the mycelial mat was dried at 40^0C 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% trifluoroacetic 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 [34].

- **Solid state fermentation:**
  Solid state (substratum) fermentation (SSF) is generally defined as the growth of the microorganism on moist solid materials in the absence or near the absence of free water. In recent years SSF has shown much promise in the development of several bioprocesses and products.Solid substrate fermentation was done by mixed cultures of different fungal strains. For better biomass and also secondary metabolite productions, the co culture of fungi during the fermentation process. By using different process parameters that can contribute the lovastatin production, the optimum levels was identified, which is carried out the solid state fermentation in conical flasks that contain optimized nutrients . There are four process parameters been used which is temperature, fermentation time, inoculums volume and pH of the solid medium were chosen for investigating and also the procedure of this process parameters was mostly contribute to the growth of the different fungal strains during solid state fermentation. In solid-state fermentation (SSF), the cultivation of *Monascus sp*. in steamed rice is very exuberant.
  Nowadays, the solid-state fermentation become the most effective ways to ferment *Monascus sp*. to gain the lovastatin because of its advantages compared to the submerged fermentation. The advantages are such as water and also energy is less used and the most important fact is that it is can produce high yield of the lovastatin. For addition, to minimize the production cost, a few efforts have been done using solid-state fermentation for the production of the lovastatin[19].

- **Effects of substrates:**
  There are reports that raw materials such as cassava starch, pear juice and also dairy milk are favourable for the *Monascus sp*. growth. There are also enhancement this substrate with the others nutrients such as vitamins and also organic nitrogen supplements. Substrates such as wheat bran, rice bran, maize flour and sorghum grain being used in the solid-state fermentation process to find the suitable substrate for maximum lovastatin production then incubated to get the yield by the HPLC analysis [19].

- **Effects of carbon and nitrogen additives**:
  Carbon and nitrogen sources is very important in the fermentation activity because this nutrients is contribute and gives major effect to the formation of biomass and the metabolite. Peptone is one of the organic nitrogen source for the lovastatin production, the lower concentration of the peptone will be increase the production of lovastatin but the higher the concentration of the peptone will decrease the production of the lovastatin. The effect of the carbon source such as glucose, maltose and also glycerol also the combination either both of the carbon source will required for the higher of the production of the lovastatin[19].

➢ **Simvastatin:**

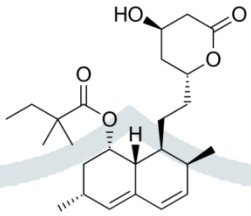

Figure 4. Simvastatin

Simvastatin is a methyl analogue of lovastatin and is synthesized from a fermentation product of *Aspergillus terreus*. Simvastatin is a no hygroscopic white crystalline powder, insoluble in water but quite soluble in chloroform, methanol and alcohol with pKa of 4.68 . The molecular weight of this compound C25H38O5 is 418.57. Simvastatin is the pharmacologically inactive lactone form of simvastatin acid, botanic acid, 2,2-dimethyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl) ethyl]-1-naphthalenyl ester. Simvastatin is a lactone which needs the opening of the ring for it to become active. Simvastatin is a crystalline powder, that practically water insoluble that is obtained as a fermentation product of *Aspergillus terreus* and is poorly absorbed from the gastro-intestinal (GI) . Therefore, it is very important to enhance its dissolution rate substantially leading to its improved bioavailability.
Currently, two semi synthetic processes are widely used to synthesize simvastatin starting from lovastatin . The commonly adapted process starts with the hydrolysis of lovastatin to yield the key intermediate monacolin J, followed by the lactonization of the acid to protect the C11 hydroxyl group and trimethylsilylation protection of the C13 hydroxyl. The protected monacolin J is then subjected to acylation by –dimethylbutyryl chloride to yield the protected form of simvastatin, which is subsequently deprotected to yield simvastatin [35].

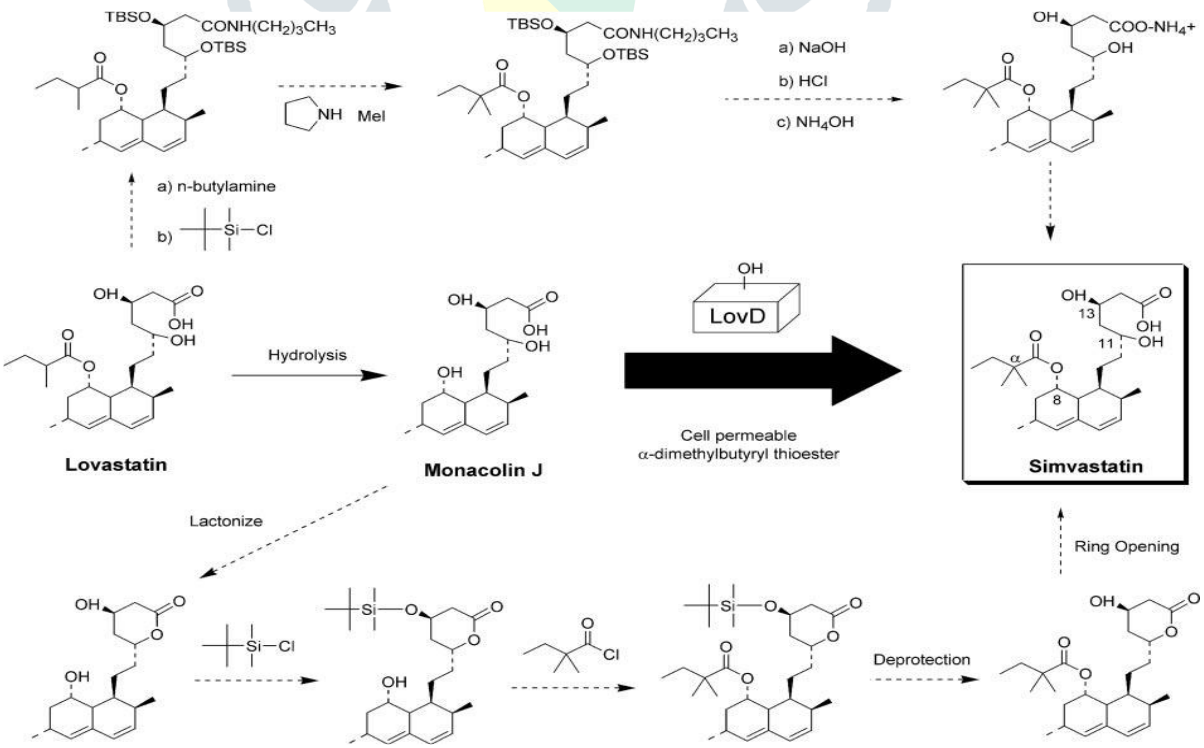

Figure 5. The bio catalytic reaction deliberate is the enzymatic conversion of monacolin J to simvastatin. LovD is able to regioselective acylate the C-8 hydroxyl group. Two commonly used semi synthetic processes are shown with dashed arrows.

- **Low-density fermentation:** E. coli BL21(DE3)/pAW31 was grown in LB medium at 37°C to an OD600 of 0.5, at which time 100μM IPTG was added to the culture, and expression was performed at RT for 16h. A 10 ml culture was transferred to a 15-ml centrifuge tube. The cells were collected by centrifugation (4,000 μg for 10 min). The cell pellet was resuspended in 957μl of the supernatant. The pH was adjusted to pH 7.9 with 1.0 N NaOH, followed by the addition of 33μl 450 mM monacolin J (final concentration, 15 mM) and 6μl pure DMB-S-MMP (final concentration, 25mM). The culture was then shaken at 300 rpm at RT. At each time point, a 4μl aliquot was removed from the reaction mixture and quenched in 300μl ethyl acetate containing 1% trifluoroacetic acid. The organic phase was removed, evaporated, and redissolved in acetonitrile for HPLC analysis.

- **High-density F1 fed-batch fermentation**: Methods for F1 fed-batch fermentation and medium composition were adopted from methods previously described. The vitamin solution is excluded from both the fermentation medium and the feed medium. A starter culture was grown overnight in 5ml of LB medium (with 35 mg/liter kanamycin) at 37°C and 250 rpm, and 1 ml was used to inoculate a 100-ml shake flask seed with F1 medium (with 35 mg/liter kanamycin). Ten milliliters of the seed F1 culture was used to inoculate a 2-literApplikon Biobundle vessel containing 1 liter of F1 medium. Fermentation was conducted at 37°C, and the pH was maintained at 7.1 throughout the experiment with 1 M H_2SO_4 and half-concentrated NH_4OH. Aeration was controlled at 0.2 to 0.4 liters/min, and agitation was maintained at 900 rpm. When the OD600 reading reached between 5 and 10, the temperature of the fermentation was reduced to RT, followed by the addition of 200 M IPTG to induce protein expression. At the same time, a peristaltic pump delivered 0.08 ml/min of the feed solution to the fermentor. Effective LovD activity and concentration at different stages of the fermentation were measured as described above. To prepare resting cells for bioconversion studies, the cells were centrifuged at 5,000 g for 10 min, followed by gentle resuspension in the same volume of phosphate-buffered saline buffer (pH 7.4). Monacolin J and DMB-S-MMP were then added to the cell aliquots to initiate the synthesis of simvastatin.

➢ **Pravastatin:**

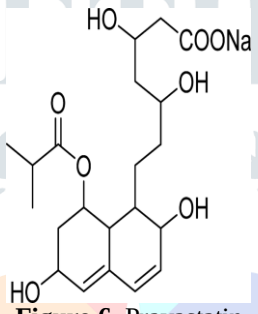

Figure 6. Pravastatin

The cholesterol-lowering blockbuster drug pravastatin can be manufactured by stereoselective hydroxylation of the natural product compactin. *Penicillium chrysogenum* can be programmed towards an industrial pravastatin production process. Following the prosperous introduction of the compactin pathway into the β-lactam–negative *P. chrysogenum* DS50662, a new cytochrome P450 (P450 or CYP) from *Amycolatopsis orientalis* (CYP105AS1) was isolated to generate the final compactin hydroxylation step. Structural and biochemical characterization of the WT CYP105AS1 reveals that this CYP is an efficient compactin hydroxylase, but that predominant compactin binding modes lead mainly to the ineffective epimer 6-*epi*-pravastatin. To avoid costly fractionation of the epimer, the enzyme was evolved to invert stereoselectivity, producing the pharmacologically active pravastatin form.

- **Strains:** *E. coli* DH10B and TOP10 (Invitrogen) were used for gene cloning. *P. chrysogenum* strains used were laboratory strain Wisconsin 54-1255 , the high penicillin producer DS17690 , the single penicillin gene cluster derivative DS47274 , and the β-lactam free platform strain DS50662 .

- **Media:** *P. chrysogenum* was cultivated for 168 h at 25°C in synthetic medium at 250 (shake flask) or 400–550 rpm (microtiter plate; MTP). To manufacture penicillin V, 2 g/L phenoxyacetic acid was added. *P. citrinum* spores were harvested from potato dextrose agar (PDA) slants in 0.5% Tween 80 and cultivated.

➢ **Construction of a Compactin Producing *P. chrysogenum* Strain:** The *P. citrinum* compactin gene cluster was cloned in three parts. The central and 3′-end (14 and 6 kb) exist readily PCR amplified and cloned using Gateway technology (Invitrogen) into entry vectors pDONRP4-P1R and pDONR221, respectively. The 18-kb fragment was cloned in two steps. First, the 10- and 8-kb fragments were cloned individually in pCR2.1 TOPO T/A (Invitrogen) and combined subsequently through compatible restriction ends. The small piece of remaining and interfering oligonucleotide sequence in *mlcA* was removed through exchange of an internal genomic PCR amplified *mlcA* fragment (4.1-kb Acc65I fragment). Ligation of all fragments produced the full-length 18-kb in pDONR41Zeo. All PCR amplified fragments were verified via sequencing. Linearized plasmids were transformed to *P. chrysogenum* as described previously. The obtained product is hydroxylated to yield Pravastatin which is characterized by using LC-MS analysis.

VI. **CONCLUSION:** Statins are proven to provide various medicinal properties like anti-cancer, bone maturation, multiple sclerosis In this view of its enormous market demands in the present study an attempt was made to review the production of natural and semi synthetic statins by fermentation technology. A detailed note on various organisms employed for the production of these statins and different fermentation techniques for the same was studied. This review also helps in understating the inhibition of HMG-CoA reducatse and various medicinal properties of statins.

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