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Review ((11234 words))

Physiological, morphological and kinetic aspects of lovastatin biosynthesis by *Aspergillus terreus*

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Abstract

This review focuses on selected aspects of lovastatin biosynthesis by *Aspergillus terreus*. Biochemical issues concerning this process are presented to introduce polyketide metabolites, in particular lovastatin. The formation of other than lovastatin polyketide metabolites by *A. terreus* is also shown, with special attention to (+)-geodin and sulochrin.

The core of this review discusses the physiology of *A. terreus* with regard to the influence of carbon and nitrogen sources, cultivation broth aeration and pH control strategies on fungal growth and product formation. Attention is paid to the supplementation of cultivation media with various compounds, namely vitamins, methionine, butyrolactone I,. Next, the analysis of fungal morphology and differentiation of *A. terreus* mycelium in relation to both lovastatin and (+)-geodin formation is conferred. Finally, the kinetics of the process, in terms of associated metabolite formation with biomass growth is discussed in relation to published kinetic models. This review is concluded with a list of the most important factors affecting lovastatin and (+)-geodin biosynthesis.

Introduction

Lovastatin belongs to the group of the organic compounds, which are commonly named statins. They are important substances for medicine as they are capable of decreasing the level of the endogenous cholesterol in the human organism and thus are used against hypercholesterolemia. This capability is connected with the feature that statins are competitive inhibitors of 3-hydroxymethyl glutaryl-CoA (3-HMG-CoA) reductase, which is responsible for the transformation of 3-HMG-CoA into mevalonate. This reaction is the early stage in the pathway leading from acetyl-CoA into cholesterol. The inhibition of the cholesterol biosynthetic pathway at this stage is more efficient and safer for humans as the accumulation of more complicated intermediates, which contain sterol rings, is avoided [1]. The research aiming at the discovery of statins in filamentous fungi was mainly performed by scientific teams in Japan and in the United States in the seventies. This history was described in detail in [2] and [3].

The most profound producers of natural statins, i.e. mevastatin and lovastatin, are *Penicillium citrinum*, *Monascus ruber* and *Aspergillus terreus* [1,2]. Other statins used contemporarily in medicine were obtained by means of enzymatic or chemical modification of the natural compounds or by chemical synthesis alone. These are, for example, simvastatin, fluvastatin, pravastatin, atorvastatin and they are produced by various pharmaceutical manufacturers [1,2].

Lovastatin is the international name of the substance, which is actually the lactone form of the natural β-hydroxy acid, called mevinolinic acid. Mevinolinic acid is the active compound in the human organism and is also excreted from the fungal cells into cultivation media. Its systematic name is 2-methyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-piran-2-yl)ethyl]-1-naphtalenylesther of butanoic acid. Its lactone, i.e. lovastatin, is formed in the course of the extraction of mevinolinic acid from cultivation media. The lactone becomes the active β-hydroxy acid again in the human alimentary canal. Although everything, which is going to be written further in this review, refers actually to mevinolinic acid as the product of *A. terreus* metabolism, for the sake of simplicity the name "lovastatin" will be used as more authors prefer it. Nevertheless, it is worth mentioning that in the earlier works other names for the lactone form of this compound are used, e.g. mevinolin [4] or monacoline K [2] or MSD803 [5].

From a microbial metabolism point of view, lovastatin belongs to the large group of secondary metabolites called polyketides. Biosynthesis of such metabolites usually consists of at least two stages. The first stage catalyzed by a polyketide synthase (PKS) is always obligatory. There are various types of polyketide synthases, which have both different structures and functions. Not to focus on details, PKSs can be divided into iterative and module synthases. The iterative PKS is a single enzyme containing between five and seven active sites of various catalytic activities such as ketoreduction, enoilreduction, dehydration, methyl group transfer, acyl group transfer, cyclization and aromatization. These

active sites can be used repeatedly in a single reaction to form an individual product molecule. The module PKS is an enzymatic complex, which fulfills the same functions, as the one mentioned above, but each active site of the enzyme is used only once in the formation of an individual product molecule. The detailed description of different types of PKSs can be found in [6] and [7].

It frequently occurs that a PKS product is further subjected to other enzymatic reactions. These reactions are called post-PKS tailoring steps and they are usually oxidations, estrifications or methylations. The detailed description of the post-PKS tailoring was presented in [8].

Lovastatin biosynthesis by *A. terreus* is performed in two stages. The first stage is catalyzed by lovastatin nonaketide synthase (LNKS, EC 2.3.1.161), which belongs to type I polyketide synthases and catalyzes the iterative nine-step formation of the polyketide [9-12].

This PKS stage leads to 4a,5-dihydromonacoline L and the stoichiometric equation can be written as:

$$\mathrm{AcCoA + 8\,malonyl\text{-}CoA + 11NADPH + 10H^{+} + S\text{-}adenosyl\text{-}L\text{-}methionine} \xrightarrow{\mathrm{EC2.3.1.161}} \xrightarrow{\mathrm{EC2.3.1.161}} \mathrm{4a,5\text{-}dihydromonacoline\,L + 9CoA + 8CO_2 + 11NADP + S\text{-}adenosyl\text{-}L\text{-}homocysteine + 6H_2O}$$

The post-PKS tailoring hydroxylation and oxidation steps are performed with the participation of molecular oxygen. The obtained intermediate monacoline J is then transestrified with (2R)-2-methylbutyric acid, which is another intermediate formed in the reaction catalyzed by lovastatin diketide synthase (LDKS). The simplified scheme of lovastatin biosynthesis is presented in Fig.1. The mechanism of lovastatin biosynthesis is quite well recognized, including the gene cluster with Open Read Frames (ORFs) coding the sequences of LNKS, LDKS, oxidizing and

transestrifying enzymes. Also the functions of other ORFs, which play important roles in lovastatin biosynthesis, were recognized. Additionally, the amino acid sequences of the enzymes engaged in biosynthesis of lovastatin are known [9]. For more details concerning the biochemical and genetic aspects of lovastatin biosynthesis papers by Kennedy et al. and Sunderland et al. can be recommended [9,10].

Although lovastatin is the most important metabolite of *A. terreus*, this microorganism of a rich secondary metabolism is also capable of biosynthesizing other compounds. It was already reported in the thirties that *A. terreus* strains were capable of formation of a substance, which was then called (+)-geodin [13]. Its systematic name is (+)-5,7-dichloro-4-hydroxy-6'-methoxy-6-methyl-3,4'-dioxomethylester of spiro(benzofuran-2(3H),1'-(2,5)-cyclohexadien)-2'-carboxylic acid and it is also a product of the polyketide pathway and, as lovastatin, is formed from malonyl-CoA and acetyl-CoA [14].

The metabolic pathway leading to (+)-geodin formation differs greatly from the lovastatin one. First of all, the PKS stage comprises the action of an uncharacterized octaketide synthase. Next, there are more post-PKS tailoring steps, which lead to the formation of such compounds as emodin, questin, sulochrin and finally (+)-geodin (Fig. 2). Other compounds as asterric acid or methyl asterrate might be formed from (+)-geodin and sulochrin [15,16].

Recent papers have reported on (+)-geodin. Askenazi et al. investigated the transcriptional and metabolite profiles of the lovastatin-producing fungi and they found significant amounts of (+)-geodin in several *Aspergilli* strains, including

lovastatin producer *A. terreus*. They also managed to disrupt (+)-geodin biosynthesis with the use of genetic engineering techniques [14].

Hargreaves et al. managed to isolate and determine the structure of (+)-geodin and several derivatives of asterric acid from *Aspergillus sp.* They also showed their antifungal properties [16]. Also Couch and Gaucher mentioned (+)-geodin in the context of the elimination of sulochrin biosynthesis [17]. Several enzymes of (+)-geodin biosynthetic pathway were also isolated and characterized [18-20].

The question whether (+)-geodin is only a by-product in lovastatin biosynthesis remains unanswered, as Askenazi et al. sustained that it is the intermediate in the biosynthesis of other natural products [14]. What is more, several scientists announced that (+)-geodin showed the activity as a glucose uptake stimulator towards rat adipocytes and enhanced the fibrinolytic activity of vascular endothelial cells [21,22].

Sulochrin [methyl 2-(2,6-dihydroxy-4-methylbenzoil)-5-hydroxy-3-metoxybenzoate], another octaketide metabolite (Fig. 2) is an antibiotic [23] and was mentioned as the co-metabolite in lovastatin biosynthesis by Schimmel et al. [24]. It is also thought to be an undesired metabolite by Couch and Gaucher, who obtained lovastatin-producing *A. terreus* mutants incapable of forming this metabolite [17]. Another important but non-polyketide metabolite of *A. terreus* is itaconic acid, which is biosynthesized by various *A. terreus* strains. Nevertheless, it is rarely mentioned in the context of lovastatin biosynthesis by the basic

lovastatin-producing strain ATCC20542. Only Lai et al. claimed that they found this metabolite, while using either lactose or glucose based media [25].

Physiological approach to investigate lovastatin biosynthesis

Influence of carbon and nitrogen sources

The choice of the relevant carbon and nitrogen sources for lovastatin biosynthesis has remained somewhat controversial since the very beginning of lovastatin history, when Monaghan et al. patented its biosynthesis process [5]. The research on lovastatin biosynthesis by *A. terreus* was performed with the use of diverse nutrients and even now it would be uncertain to claim that there is an established medium composition for this process, even with regard to carbon and nitrogen source.

The diversity of nutrients for lovastatin biosynthesis can be noticed in the patent by Monaghan et al., who proposed glucose, oat meal, corn steep liquor, tomato paste, starch or yeasts in the seed culture and lactose, yeast extract, malt extract or dextrose in the cultivation medium [5]. The early publications did not focus on the aspects of the influence of nutrients on lovastatin biosynthesis [4,26].

Reviewing the influence of nutrients on lovastatin biosynthesis, it is worth mentioning with the work of Szakacs et al., who tested lactose with corn steep liquor and sucrose with corn steep liquor for the Hungarian strain *A. terreus* TUB F-514 [27]. It was observed that the highest lovastatin titer of 400 mg l^{-1} was obtained with the use of lactose as the carbon source. Lovastatin titers on sucrose were 40% of that on lactose.

One of the most detailed experiments concerning media composition for lovastatin production was performed by Hajjaj et al. [28]. They tested the growth of ATCC74135 strain on synthetic and complex media. Glucose and sodium glutamate were applied in the synthetic media, whereas lactose or glucose, peptonized milk (also lactose source) and yeast extract in the complex media. In the synthetic media, fast glucose utilization was observed from the level of 45 g l^{-1} down to zero within 50 hours with the specific glucose uptake rate of 0.196 g g^{-1} h^{-1}. Lactose was then far slower assimilated and even not ultimately exhausted: from 50 g l^{-1} to about half of this concentration within 300 hours. Nevertheless, when glucose was being assimilated, a side effect was observed. Ethanol was excreted, up to 1.3 g l^{-1} in the phase of fast glucose uptake. Later on, this ethanol was reutilized. The same phenomenon was observed by Bizukojc and Ledakowicz in the experiments with ATCC20542 strain [29]. Glucose and yeast extract were used at concentrations of 45 g l^{-1} and 12.5 g l^{-1}, respectively. The maximum ethanol concentration reached 10 g l^{-1} at glucose depletion.. Although ethanol was further utilized, as it had been observed by Hajjaj et al. [28], it did not contribute to lovastatin formation. Lovastatin concentration did not increase after glucose depletion. Additionally, Bizukojc and Ledakowicz claimed that unidentified fruity-smelling compounds, probably terpenes, were simultaneously produced. When glucose was replaced with lactose at an initial concentration of 20 g l^{-1} neither ethanol nor other fragrant compounds were formed [29]. These data suggest that slowly degradable carbon sources such as lactose are favorable for lovastatin production. It is also worth mentioning that Hajjaj et al. obtained good

lovastatin yields with the use of a glucose and lactose mixture in the experiments, in which they applied peptonized milk (the source of lactose) as the nitrogen source with glucose as the carbon source (Table 1) [28].

The hypothesis that slowly degradable carbon sources are preferred in lovastatin production is also claimed by Casas Lopez et al. [30]. They used the basic strain ATCC20542 and tested fructose, glycerol and lactose as the carbon sources. These authors never used glucose. They believe that it is the invalid carbon source for lovastatin biosynthesis by *A. terreus* as it causes catabolic repression. Analyzing substrate uptake rate, Casas Lopez et al. found that, irrespective of the nitrogen source used, fructose was the fastest utilized carbon source, followed by glycerol and lactose [30]. Apart from the proper choice of carbon and nitrogen source they drew an attention to another issue, i.e. carbon to nitrogen (C/N) ratio. The increase of C/N ratio in the idiophase even up to 41.3, which was achieved by the feeding of the culture with the carbon source, led to an elevated lovastatin yield (Table 1) [30].

The systematic approach in testing the influence of the initial carbon source concentration on lovastatin biosynthesis was performed by Bizukojc and Ledakowicz [31]. In this work lactose was used as the carbon source and yeast extract as the nitrogen source. It was shown that lovastatin biosynthesis was strongly limited by lactose (Fig. 3). At the initial lactose concentrations of 5 and 10 g l^{-1}, lovastatin formation ceased as a result of lactose depletion. However, the increase in the initial lactose concentration from 20 to 40 g l^{-1} did not result in any significant increase in lovastatin titer. Furthermore, lactose was not exhausted at

its higher initial concentration of 40 g l^{-1} [31]. Similar results were found by Hajjaj et al. [28]. Upon these observations it can be assumed that *A. terreus* cultivation in the carbon source fed fed-batch culture would prove the efficient method to perform lovastatin production. This issue shall be discussed further.

One of the most important issues connected with any microbial cultivation is to test, if there is a chance to apply the mineral nitrogen sources. Complex nitrogen sources are not welcome in the industrial processes as their chemical composition is not always reproducible and they are at times expensive. Hajjaj et al. investigated this aspect of lovastatin biosynthesis in detail, testing inorganic nitrogen sources and amino acids [28].

Generally, amino acids are utilized by fungi only as the nitrogen source because no ammonium ions are found in the broth. *A. terreus* responds similarly and the optimum amino acids to be utilized by this fungus for lovastatin biosynthesis was either sodium glutamate or histidine. Several tens of mg lovastatin per liter was biosynthesized, if these amino acids were applied [28]. On the other hand, Hajjaj et al. found that *A. terreus* could utilize inorganic and organic ammonium salts as ammonium tartrate or nitrates for biomass growth. Unfortunately, in such media no lovastatin biosynthesis was observed because such nitrogen sources, when being assimilated, acidify the broth [28]. Furthermore, Lai et al. mentioned that ammonium sulfate was not suitable for lovastatin production in their experiments performed to obtain data for the statistical medium design [32]. Therefore, even if synthetic media were to be used

for lovastatin production, at least one amino acid as a nitrogen source should be present.

The complex nitrogen source for lovastatin biosynthesis by *A. terreus* has a wide selection: yeast extract, corn steep liquor, soybean meal, oat meal, peptonized milk are the most frequently used [*inter alia:* 28,30,31,33,34]. Due to the fact that these are mixtures of various organic nitrogen compounds, mainly amino acids, the detailed investigation on their influence on lovastatin biosynthesis can only be quantified using organic nitrogen concentration as the analytical parameter.

Casas Lopez et al. noticed that lovastatin biosynthesis took place in the nitrogen-limited phase during cultivation and that starvation, with regard to organic nitrogen, induced *A. terreus* to produce lovastatin [30]. Within contrast Hajjaj et al. claimed that lovastatin formation was observed only when lactose was depleted. It was concluded that lactose starvation was favorable for lovastatin biosynthesis [28]. Bizukojc and Ledakowicz, having systematically tested the influence of both lactose and yeast extract on lovastatin production, found that lactose consumption was unquestionably required for lovastatin production (Fig. 4), starting in biomass growth phase and continuing into the idiophase [31]. It was the organic nitrogen, which inhibited lovastatin biosynthesis in batch processes and its initial level should not be too high (Fig. 4).

To sum up the issues concerning the influence of C- and N-sources on lovastatin production by *A. terreus*, various approaches to the formulation of medium composition and resulting lovastatin titers were collected in Table 1.

The aforementioned considerations focused only on the effect of the media composition on lovastatin formation. But lovastatin is not the sole metabolite produced by *A. terreus*. As it was mentioned in the introduction, some authors have referred to other metabolites, as (+)-geodin, produced by this fungus [14-16] and only Bizukojc and Ledakowicz tested the influence of media composition and process conditions on simultaneous lovastatin and (+)-geodin biosynthesis [35,36].

According to Bizukojc and Ledakowicz (+)-geodin biosynthesis is more dependent on the initial lactose concentration than lovastatin [35,36]. They observed it only at higher initial lactose concentrations of 20 and 40 g l^{-1} (Fig 5) and the presence of lactose in the late idiophase in the broth was necessary to start and prolong its formation. Similar results were obtained in fed-batch cultivations (Fig. 6). The depletion of lactose eventually ceased (+)-geodin formation. Furthermore, (+)-geodin biosynthesis was also more sensitive towards organic nitrogen concentration as the inhibitive effect was in this case stronger (Fig. 5). Under nitrogen starvation conditions (+)-geodin biosynthesis was the most efficient [35,36].

The issue of establishing feeding strategies in fed-batch cultivations is also closely connected with the choice of carbon and nitrogen sources and their concentrations. Some authors proved that carbon source feeding was favorable for lovastatin biosynthesis. Novak et al. used repeated fed-batch culture with glucose as the carbon source. As the depletion of glucose ceased lovastatin formation, they fed the culture twice with glucose solution, achieving elevated lovastatin yield by

about 37%. They also noticed that if the feeding was repeated more times, it did not assure increased lovastatin productivity [37].

The strategy applied by Sitaram Kumar et al. was different as there were different carbon sources in the batch medium and in the feed. The batch medium contained glucose, maltodextrin and starch, while the feed contained only maltodextrin. Their culture was fed from 72 hour of the run in 5 hour intervals. Interestingly the nitrogen source, in the form of corn steep liquor was also fed into the bioreactor but in a less frequent manner, every 24 hours. The latter might be controversial as nitrogen is believed to inhibit lovastatin formation. Nevertheless, lovastatin volumetric productivity was almost doubled due to this feeding strategy [33].

Bizukojc and Ledakowicz tested the effect of lactose feeding in the discontinuous fed-batch culture on lovastatin and (+)-geodin formation by *A. terreus* (Fig. 6). It was concluded that lactose feeding increased lovastatin titer [31,35]. Furthermore, it was observed that (+)-geodin production was increased significantly by lactose feeding, in both shake flask and bioreactor cultivations. This was attributed to an elevated lactose concentration in the late idiophase [35,36].

The most detailed research on feeding strategies in lovastatin biosynthesis was performed by Rodriguez Porcel et al. They tested the following feeding strategies: either complete medium, medium without organic nitrogen or only minerals without carbon and nitrogen. Their processes were not typical fed-batch cultivations but rather semi-continuous runs because biomass-free medium was

retrieved from the bioreactor during the feeding phase. The nitrogen free medium was observed to be the optimum feed, which increased lovastatin production by 315% [38].

Influence of other compounds on lovastatin biosynthesis

Apart from the main nutrients, some authors have focused on the supplementation of *A. terreus* cultivation media with special compounds added at low concentrations from several to tens of mg per liter. The main motivation for this approach is connected with the biochemical mechanisms governing lovastatin biosynthesis, to be more precise the action of lovastatin nonaketide synthase (see the stoichiometric equation). For example, precursors of NADP or CoA might have had an impact on the process. A similar action can be expected in relation to methionine, which is a component of the methyl group donor S-adenosyl-L-methionine, in the third step of LNKS action [9].

The effect of methionine supplementation was tested by Lai et al. They claimed that when DL-methionine (100 mg l^{-1}) was added at 72 hour of cultivation, lovastatin formation increased by 20%. In contrast L-methionine did not exert such an effect [32]. They proposed two hypotheses to explain this: either D-methionine also plays a role in the transfer of methyl groups or the racemate activates the polyketide synthase. It is controversial hypothesis, as Kyoto Encyclopedia of Genes and Genomes (KEGG) does not indicate any function of D-methionine in the metabolic maps. Nevertheless, L-methionine

racemase, whose cofactor is pyridoxal-5'-phosphate, is mentioned both in KEGG and Braunschweig Enzyme Database (BRENDA) as EC 5.1.1.2.

Bizukojc et al. tested the effect of B-group vitamins supplementation on lovastatin biosynthesis. Not only precursors of NADP (nicotinamide) and CoA (calcium pantothenate), but also thiamine, riboflavin and pyridoxine were taken into account. All these vitamins, excluding thiamine, increased the lovastatin maximum volumetric formation rate. Calcium pantothenate increased both lovastatin on lactose ($Y_{LOV/LAC}$) and lovastatin on biomass ($Y_{LOV/X}$) yield coefficients and so did pyridoxine (Table 2) [39]. Bizukojc et al. also observed that lactose utilization was decreased during the middle and late idiophase when B-group vitamins were present in the medium. Because lovastatin biosynthesis is lactose-dependent, as it was mentioned above, reduced lactose utilization and resulting elevated lactose concentration contributed to increased lovastatin formation. These results were further enhanced with the supplementation of the vitamin mixture. It increased volumetric and specific lovastatin formation rates, as well as yield coefficients $Y_{LOV/LAC}$ and $Y_{LOV/X}$ (Table 2) [39]. In terms of (+)-geodin production, results were different. Both nicotinamide or riboflavin did not increase the maximum (+)-geodin formation rate. However (+)-geodin on lactose yield coefficients ($Y_{GEOD/LAC}$) were higher in all cases with vitamin supplementation. During this study (+)-geodin on biomass yield coefficients ($Y_{GEOD/X}$) were not calculated as they have no biological sense, if the definition of yield coefficient is taken into account (Table 2). (+)-geodin formation was determined to be non-growth associated. This will be discussed further.

Another supplementary medium component was proposed by Schimmel et al. [24]. They cultivated *A. terreus* ATCC20542 on glucose, lactose, yeast extract and peptonized milk and in addition applied butyrolactone I [α-oxo-β-(*p*-hydroxylphenyl)-γ-(*p*-hydroxy-*m*-3,3-dimethylallyl-benzyl)-γ-methoxycarbol-γ-butyrolactone]. Butyrolactone I is a secondary metabolite produced by *A. terreus*. The motivation for the use of the surplus dose of this metabolite was that this compound is known to act as a self-regulating factor in some bacteria. With respect to *A. terreus*, it, first of all, had an impact on the morphology of the fungus, increasing the amount of the branched hyphae, when added at 8 hour of the run. Secondly, it increased the number of spores occurring in the submerged sporulation. Finally, lovastatin yield was almost tripled (up to 940 mg l^{-1}), if butyrolactone I was added at 100 μM at 120 hour of the run. Nevertheless, sulochrin formation was also higher by about 82%. The best results were obtained, if butyrolactone I was added in the early idiophase, at the completion of the exponential phase [24].

Influence of aeration rate and oxygen concentration on lovastatin biosynthesis

The aeration rate is one of the key factors influencing filamentous fungi growth and there are several papers, which focus on this aspect of lovastatin biosynthesis by *A. terreus*. Nevertheless, it is difficult to find the unanimous statements concerning the influence of aeration rate and oxygen saturation of the medium on lovastatin biosynthesis as different approaches to test the effects of oxygenation were used. Also, most of the information comes from three teams,

who obtained, to a certain extent, contradictory results, probably due to different experimental procedures applied.

The interesting idea to test the influence of oxygen on lovastatin biosynthesis was proposed by Casas Lopez et al. [40]. Their paper concerned the optimization of medium composition for lovastatin production. It is known that for tens experiments to perform, while using response surface methodology or other statistical optimization methods, hardly can the influence of oxygen be tested due to the fact that such experiments are made in shake flasks not in bioreactors. Casas Lopez et al. solved this problem using a controlled atmosphere chamber with the aeration gas mixtures containing 20, 50 and 80% (v/v) oxygen, respectively, for the cultivation of ATCC20542 strain. They found that the aeration gas enriched with oxygen strongly increased lovastatin formation. These preliminary results were later confirmed by the same researchers, who found the positive effect of high aeration rate of the medium on lovastatin biosynthesis in the bubble column [41]. They worked at three vvm values 0.5, 1.0 and 1.5 l_{air} l^{-1} min^{-1} and the gas phase was either air or air enriched with oxygen at the level of 80% (v/v). At vvm=1 l_{air} l^{-1} min^{-1} and 80% (v/v) oxygen, high lovastatin titers were obtained, up to 300 mg l^{-1}. Upon this, they claimed that in the bubble column it was insufficient to aerate the medium with air alone, as on average even five-fold increase of lovastatin concentration could be achieved when air enriched with pure oxygen was used [41]. Unfortunately, these authors supplied no information about the changes of dissolved oxygen concentration in the medium in the dependence on the aeration gas composition used. The only data that could make

the issue of oxygen utilization clearer were the changes of specific oxygen uptake rate (SOUR). The SOUR decreased with time showing approximately the same profile from about 1 mmol O_2 g^{-1} h^{-1} in the early hours of the process down to about 0.1 mmol O_2 g^{-1} h^{-1} at about 200 hour, irrespective of the fact, whether there was 80% (v/v) of oxygen in aeration gas or not. Thus, it was unclear what mechanism was responsible for the better lovastatin formation under oxygen enriched conditions. Rodriguez Porcel et al. claimed that oxygen was required in the post-PKS stage of lovastatin biosynthesis [41]. Unfortunately, these authors did not refer to the formation of octaketide metabolites, as sulochrin or (+)-geodin, which require molecular oxygen in two or three post-PKS steps (Fig. 2).

Lai et al. tested a mutant which originated from ATCC20542 strain and showed in their experiments that the oxygen saturation profile was quite typical in the cultivation of *A. terreus*. There was a fast decrease within the first 24 hours, which reflected the exponential growth of biomass. When the dissolved oxygen level reached 30%, a cascade control using agitation was used to maintain the saturation level. If uncontrolled, the dissolved oxygen might have decreased down to zero before the idiophase [42].

Hajjaj et al. compared volumetric oxygen uptake rates (OUR) in the aforementioned complex and synthetic media. It occurred that in the complex media OUR exceeded 75 mmol O_2 l^{-1} h^{-1} in the end of trophophase, while in the synthetic media it was not higher than 18 mmol O_2 l^{-1} h^{-1}. Lovastatin formation was higher in complex media, however there was no proof that it was due to higher oxygen uptake and not to nutrient composition [28]. Novak et al. claimed

that the optimum oxygen saturation in the batch culture with glucose as a carbon source was 70%. Either too high (80%) or too low (35%) did not assure good lovastatin titer [37].

Bizukojc and Ledakowicz tested lovastatin biosynthesis in batch and fed-batch processes using three constant aeration rates [36]. Within contrast to Casas Lopez et al. [34], it was found that higher aeration rate enhanced (+)-geodin, instead of lovastatin formation in batch cultures (Fig. 7). Higher aeration rate increased organic nitrogen uptake rate and the decrease of pH in the early idiophase was faster. Final (+)-geodin concentration was ten times higher, when vvm was increased from 0.308 to 0.513 l_{air} l^{-1} min^{-1}. Lovastatin titer was then four-fold lower. When aeration rate was increased further (up to 1.026 l_{air} l^{-1} min^{-1}) in the lactose-fed fed-batch culture, the final (+)-geodin titer exceeded 200 mg l^{-1}, while lovastatin concentration decreased to less than 10 mg l^{-1} [36].

This effect of aeration was also seen when one compared the rotary shake flask culture run with bioreactor runs. Bizukojc and Ledakowicz found that at YE_0= 2 g l^{-1} and LAC_0= 20 g l^{-1} yield coefficient $Y_{GEOD/LAC}$ was equal to 3.76 mg GEOD g LAC^{-1} in shake flask culture and 12.3 mg GEOD g LAC^{-1} in the bioreactor at vvm=0.308 l_{air} l^{-1} min^{-1}. At the same time yield coefficient $Y_{LOV/LAC}$ was equal to 4.34 mg LOV mg LAC^{-1} and 4.83 mg LOV mg LAC^{-1} in the corresponding shake flask and bioreactor runs [35,36]. It is clear that the aeration in the shake flask culture is poor compared to that of a stirred tank bioreactor.

The elevated (+)-geodin titers induced by the increase of aeration rate were probably due to the fact that in the post-PKS tailoring steps leading to (+)-geodin

formation there is a higher demand for molecular oxygen. Emodin anthrone oxygenase, questin oxygenase, dihydrogeodin oxidase are the enzymes requiring the molecular oxygen as a cofactor in the (+)-geodin biosynthetic pathway. In the lovastatin post-PKS pathway there is one hydroxylation and one oxidation step. Only the latter requires molecular oxygen [36].

A completely different approach to investigate the effect of oxygen on lovastatin biosynthesis was applied by Lai et al. [43]. They tested the influence of the organic oxygen carriers on the process. Such oxygen carriers are usually liquid n-alkanes of 12 to 16 carbon atoms in the chain.

A 2.5% addition of dodecane to the medium in shake flask culture increased lovastatin titer from 100 up to 400 mg l^{-1}. The adverse effect was observed in a bioreactor, where the 4-fold decrease of lovastatin concentration was found [43]. Lai et al. found that the addition of dodecane increased dissolved oxygen of up to 70%, while in the dodecane-free run it was on the level of 20%. Probably, in this case the effect was similar to the one reported by Bizukojc and Ledakowicz [36]. The higher oxygen saturation in the medium did not facilitate lovastatin formation in the bioreactor. Nevertheless, Lai et al. [43] reported nothing concerning the by-products of either sulochrin or (+)-geodin, so the effect of organic oxygen carriers on these metabolites cannot be discussed.

The most important information on the influence of oxygen on the formation of secondary metabolites in *A. terreus* are summarized in Table 3.

Influence of pH control

There is the general agreement concerning the influence of initial pH of the medium on lovastatin production [*inter alia*: 25,28,36,42,43]. These researchers started their cultivations with a pH at approximately 6.5. Nevertheless, the obtained pH profiles observed within *A. terreus* growth as well as the opinions on pH control were different. This is attributed to the different cultivation conditions used, e.g. shake flask vs. bioreactor, aeration rate and the application of various carbon and nitrogen sources, all of which would influence the pH profiles in *A. terreus* cultures.

Lai et al. used lactose (50 g l^{-1}), yeast extract (10 g l^{-1}) and soybean meal (1.5 g l^{-1}) in shake flask cultures and observed that pH initially decreased to below 5 within 48 hour of the run. In the bioreactor runs it took only 24 hours for the pH to drop to 5. Thereafter, the pH increased up to 8 in both systems [42].

The extent, to which the use of various nutrients can influence pH profiles was shown by Lai et al. [25]. It was observed that pH profiles differed in medium containing glucose in comparison to lactose. In lactose media pH initially increased to 7 and subsequently a decrease was observed. If glucose was used, pH decreased initially to the levels below 5.5 and than increased up to 6 [25].

Lai et al. also performed a wide range of experiments testing the influence of culturing conditions, including aeration and pH-control on lovastatin biosynthesis using lactose (70 g l^{-1}) and yeast extract (8 g l^{-1}) as nutrients. They compared pH profiles in relation to the aeration of the medium at various set

dissolved oxygen levels [44]. No direct correlation was found but all profiles looked similar; initial pH increased up to 7.6, thereafter a decrease down to the different constant values between 6.5 and 7.2 was observed [44].

The experiments with pH control using hydrochloric acid and sodium hydroxide in the production phase (about 48 hour) were not successful [44]. Setting pH at three different levels 5.5, 6.5 and 7.5 did not supply any satisfactory results as lovastatin titer, biomass content and biomass growth rate were lower in all these runs in comparison to the pH-uncontrolled run.

The more promising results concerning pH control in lovastatin production were obtained by Bizukojc and Ledakowicz [36]. They used the same nutrients as Lai et al. [44] but at lower nutrient concentrations (lactose 20 g l^{-1} and yeast extract 2 and 4 g l^{-1}). Also, pH control was started as soon as 24 hours and set at the levels of 7.6 and 7.8, depending on the initial yeast extract concentration. Owing to this procedure pH did not drop in the idiophase (Fig. 8). As a result of pH control lovastatin formation was practically unchanged and (+)-geodin formation was strongly aggravated. The important fact was that only the solution of sodium and potassium carbonates was used for pH control. The use of carbonates can be profitable as several reactions of the primary metabolism of *A. terreus*, including the one, which forms malonyl-CoA, are hydrocarbonate dependent [36].

Morphology and differentiation of *Aspergillus terreus* mycelium in the submerged culture

Morphology of the mycelium is often the key factor influencing the formation of secondary metabolites in various filamentous fungi [45].

In the submerged culture filamentous fungi grow either as dispersed hyphae or as pellets. Both forms have been reported to be present simultaneously in a bioreactor [45]. Therefore, one often discusses which morphological form is more desired and effective in the biosynthesis of a given metabolite, either dispersed or pellets. In case of lovastatin the situation is clear. Pellets dominate in the submerged cultivation of *A. terreus* and assure better lovastatin titer. All authors cited so far, who mentioned anything about *A. terreus* morphology, claimed that this fungus formed macroscopic pellets [*inter alia:* 32, 34, 37].

There are three known mechanisms of pellet formation in filamentous fungi. Either the spores aggregate in the early stages of evolution forming the pellets from the hyphae growing out of the aggregate or the pellets grow out of a single spore or hyphae evolved out of single spores, aggregate to form a more dense structure of clumps and pellets [46].

In case of *A. terreus* the first mechanism applies and the following correlation between the number of spores introduced to the preculture (n_{spores}) and the number of pellets ($n_{pellets}$) was found:

$$n_{pellets} = 4.48 \cdot 10^{-6} \left(\pm 2.85 \cdot 10^{-7} \right) \cdot n_{spores} \qquad (1)$$

and it is valid in the range of n_{spores} from $1.39 \cdot 10^9 \pm 2.89 \cdot 10^8$ l^{-1} to $2.56 \cdot 10^{10} \pm 9.64 \cdot 10^9$ l^{-1} [47].

The basic parameter to describe the morphology of the pellet-growing fungi is pellet diameter. Because fungal pellets can be either smooth or hairy, another morphological measure is also applied to evaluate pellet morphology. It is the filament ratio, which is defined as the ratio between the areas of the peripheral hairy surface of the pellet and the total area of the pellet [48]. Filament ratio represents the extent to which free filaments grow out of the dense structure of the pellet. This parameter was widely used by the researchers from University of Almeria (Spain), who dealt with the changes of *A. terreus* morphology in lovastatin biosynthesis [34,41,48,49,50]. Their research was, however, directed more into the correlation of morphology with the aeration of the medium and its rheological properties than with lovastatin yield. In our opinion the influence of the morphology on the rheological properties of the media is beyond the scope of this review and will not be discussed further. Only the hyphal morphology alone and its correlation with lovastatin formation, if found, will be discussed.

Rodriguez Porcel et al. [48] tested the morphology of *A. terreus* in a fluidized bed reactor (FBR) as well as a stirred tank bioreactor (STB). In FBR pellets had higher diameters of up to 2.5 mm, while in STB only 0.5-1 mm pellets were observed. This was obviously attributed to the increased shear forces in the STB. However, if the culture was aerated with oxygen-enriched (80% v/v) gas phase even in the STB, pellet diameter exceeded 2 mm. The filament ratio decreased from 1 down to 0.4, irrespective of the bioreactor, excluding the runs in

STB with higher agitation (800 rpm). In that case pellets got smoother and filament ratio was about 0.2. No direct correlation between pellet diameter, filament ratio and lovastatin titer was presented [48].

In other experiments Rodriguez Porcel et al. [41] started their cultivations in the bubble column with the initial spore concentration of $3.5 \cdot 10^9$ spores per liter and observed the decrease of the filament ratio from about 1 down to 0.4, irrespective of the aeration rate, initial nitrogen concentration and the enrichment of gas phase with oxygen up to 80% (v/v). Unlike filament ratio, pellet diameter occurred to be dependent on the amount of oxygen supplied. Even with the increased addition of nitrogen source but at air aeration pellet diameter of only 1.5 mm in the idiophase were obtained, while under oxygen enriched conditions mean pellet diameter exceeded 3 mm. Thus, according to Rodriguez Porcel et al., the elevated oxygenation of the culture increased pellet size and lovastatin titer (see previous chapter). However, it cannot be unequivocally claimed that higher lovastatin titers were obtained in response to pellet size or other process conditions such as oxygenation [41].

Sainz Herran et al. (2008) also proposed ultrasound sonication of the broth as a method to control fungal morphology in *A. terreus* cultivation [49,50]. The application of ultrasounds allowed for substantial changes in the morphological characteristics of hyphae. At a sonication power input higher than 556 W m^{-3} *A. terreus* ceased to grow as pellets and evolved into dispersed hyphae. Ultrasounds had no influence on biomass yield or growth rate but negatively affected

lovastatin yield decreasing it down to 28% of the value obtained in the control runs (non-sonicated) [50].

Casas Lopez et al. used a relatively old 48 hour inoculum with large diameter pellets (1.2 mm) [34]. They aimed to determine the effect of various hydrodynamic conditions on pellet size in the stirred tank bioreactor. Pellets increased in size (between 2 and 2.5 mm) under low agitation (300 rpm). At higher agitation the pellets initially grew to 1.5 mm. Thereafter pellet diameter was observed to reduce in size (0.9 mm) and was attributed to shear stress. No direct quantitative correlation between mycelial morphology and lovastatin production was shown in these experiments. Casas Lopez et al., however, claimed that the loose pellets of more filamentous morphology assured better lovastatin titers in comparison to the denser smaller pellets [34].

In contrast Gupta et al. claimed that the middle sized pellets of diameter, from 1.8 to 2 mm, were optimal for lovastatin biosynthesis [51]. They also claimed that growing external cells were responsible for lovastatin biosynthesis, although they showed no direct evidence of this. Despite this, this correlation was confirmed by Bizukojc and Ledakowicz, and will be presented further [47].

The number of spores introduced to the preculture is one of the most sensitive parameters influencing the morphology of hyphae and biosynthesis of secondary metabolites [52]. Tucker and Thomas observed that the increase of the number of *P. chrysogenum* spores above $5 \cdot 10^8$ per liter caused the transition from pelleted to dispersed growth [52]. Hyphal morphology and xylanase biosynthesis

by *A. awamori* in relation to the initial number of spores was also investigated by Smith and Wood [53].

In *A. terreus* similar relations were observed by Bizukojc and Ledakowicz [47]. The general rule was confirmed that the more spores that were introduced resulted in more pellets being formed at a reduced pellet diameter [47,52]. When the initial number of *A. terreus* spores in the preculture was equal to $2.56 \cdot 10^{10}$ l^{-1} the obtained pellets had on average diameter three times lower than for the case when only $1.39 \cdot 10^{9}$ spores per liter were introduced (Fig. 9a). The size of pellets influenced lovastatin formation. The smaller pellets were more efficient for lovastatin biosynthesis (Fig. 9a). A correlation was not clear for (+)-geodin formation until after 120 hours of cultivation and seemed to be independent of the initial number of spores and subsequently pellet diameter. At this time it became clear that (+)-geodin production was enhanced under conditions with a low spore concentration [47].

In the research concerning the morphology of fungal mycelium one can go further than to measure size and shape of the filaments or pellets. The differentiation of hyphae into apical, subapical and hyphal cells is another important feature of the fungal growth, which has an impact on metabolite formation. Whereas there are a lot of experimental data about the differentiation of mycelium in the fungi which usually grow freely dispersed, such as *P. chrysogenum*, or other filamentous organisms (*Streptomyces* sp.), the research concerning pellet-forming fungi was less frequently performed, as it is more complicated from a methodological point of view [45].

There are few papers which focus on the differentiation of the pellets formed by filamentous organisms [54-56]. The work of Hamanaka et al. mainly focused on the visualization techniques of intracellular lipids in the pellets of *Mortierella alpina* [55] and Park et al. described the visualization technique for the quantification of the intrastructure of tylosin-producing *Streptomyces fradiae* pellets [54]. Freudenberg et al. supplied some details about the differentiation of hyphae in *A. awamori* pellets. They distinguished two zones in the pellets, growing and non-growing, with the use of a fluorescent stain, acridine orange [56].

For a lovastatin producer namely *A. terreus* Bizukojc and Ledakowicz investigated the differentiation of pellets [47]. The pellets were stained with methyl blue and, having been processed, sectioned into thin slides. Two zones were distinguished and quantitatively evaluated as zone fractions. They comprise of growing Z_1 (apical) and non-growing Z_2 (hyphal) cells (Fig 9b and 9d). The growth of the active zone already started, when germ tubes emerged from spore agglomerates in the preculture (Fig. 9b). In that time growing cells fraction Z_1 increased and the kernel of the pellet (hyphal cells fraction Z_2) was rather small. Since the transfer of the evolved pellets into the cultivation medium, the fraction of the active zone Z_1 decreased and the hyphal internal part of the pellets Z_2 (kernel) dominated (Fig. 9b). Thereby, the metamorphosis of active cells into the hyphal cells took place. Also these were the hyphal cells, which finally contributed to the increase of biomass concentration expressed as dry weight, which was proved by the linear correlation shown in Fig. 9b [47].

Bizukojc and Ledakowicz claimed that specific lovastatin and (+)-geodin formation rates correlated with active zone fraction. They found that specific lovastatin formation rate decreased linearly with the decrease of active, apical zone fraction Z_1, while specific (+)-geodin formation rate increased exponentially (Fig. 9c).

Biomass growth and product formation kinetics in *Aspergillus terreus*

The kinetic modeling of lovastatin biosynthesis was seldom the object of research. Many authors showed only the graphs with the changes of lovastatin concentration in time. However, upon analyzing these data, one may conclude that lovastatin biosynthesis, unlike many other secondary metabolites, is varied in terms of growth and non-growth association i.e. mixed-growth associated. The inspection of the graphs in [27,30,33,41,44] clearly showed that lovastatin production started already in the biomass growth phase (trophophase) and was prolonged into the idiophase, especially if there was enough carbon source left in the medium or a feeding strategy was applied. Only Hajjaj et al. showed the graph, in which lovastatin appeared in the medium in the late idiophase and thus an impression may have occurred that its formation was non-growth associated [28].

The detailed analysis of product growth association was made upon the time evolution of product and biomass volumetric formation rates, as well as upon the relation between specific biomass and product formation rates [31, 35]. At a glance, lovastatin formation was mainly associated with biomass growth.

Although, the cease of biomass growth did not prevent lovastatin formation, if carbon source was available (Fig. 6). Bizukojc and Ledakowicz showed that the maxima of biomass and lovastatin volumetric formation rates did not ideally coincide (Fig 10a) and the delay in lovastatin formation in the trophophase was observed. The relation between specific lovastatin formation rate and specific biomass growth rate was not linear either (Fig 10b). The reason of this phenomenon was that at the highest biomass growth rates lovastatin is not produced due to the high nitrogen levels (nitrogen inhibition). This lag in lovastatin formation with the increase in initial nitrogen content is noted in Fig 3. Thus, one sometimes could have an impression that lovastatin formation is delayed till as late as the idiophase, which in the extreme case may provoke the conclusion that lovastatin formation is practically non-growth associated. In fact, it is mixed-growth association and this way the real reason of this phenomenon connected with elevated nitrogen level in the beginning of the process may remain unnoticed [35].

In case of (+)-geodin, the situation is completely different. Bizukojc and Ledakowicz observed that this metabolite was excreted extensively in the idiophase and there was no association between its formation and biomass growth. The yield coefficient defined as the increment of product divided by the increment of biomass was equal to infinity for (+)-geodin [36].

There are only two papers published, which focused on the kinetic modeling of *A. terreus* growth and lovastatin formation. Liu et al. proposed a morphologically structured model for biomass growth, hyphal differentiation and

lovastatin formation by *A. terreus* [57]. This model was very similar to the morphologically structured model previously shown for penicillin biosynthesis [56]. Liu et al. assumed the inhibitive effect of glucose originated from the enzymatic hydrolysis of starch, the carbon source used, but omitted the inhibitive effect of organic nitrogen, which is rather a mistake [57]. Additionally, they did not supply their own data on the hyphal differentiation but used the kinetic parameters from *P. chrysogenum* [58]. The assumption that these parameters are close to that of *A. terreus* is inaccurate and could lead to erroneous results. There is no evidence that the differentiation kinetics and morphological form of both fungi are similar. As mentioned in the previous section, *A. terreus* is known to evolve in the form of macroscopic pellets, while *P. chrysogenum* favors dispersed hyphae. Liu et al. failed to mention the morphological form of the fungus in their experimental runs [57].

Bizukojc and Ledakowicz proposed a simple unstructured kinetic model for lovastatin biosynthesis upon the following assumptions evolved from the detailed research on the influence of the initial lactose and yeast extract concentrations on the process. Thus lactose was the sole carbon source and yeast extract the sole organic nitrogen source. The excess of organic nitrogen exerted an inhibitive effect on lovastatin biosynthesis and lactose uptake. Lovastatin biosynthesis was mixed-growth associated, so lovastatin balance consisted of two terms: biomass growth associated and biomass non-growth associated lovastatin formation. Lactose was both utilized for biomass formation and lovastatin biosynthesis [31].

The following equations were proposed [31]. Here, for the sake of brevity the equations are shown in the form of specific growth rates for lactose (σ_{LAC}), organic nitrogen (σ_N), lovastatin (π_{MEV}) and biomass (μ):

$$\sigma_{LAC} = -\frac{1}{Y_{X/LAC}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC} \cdot c_X} \cdot \frac{c_N}{c_N + K_N \cdot c_X} \cdot \frac{K_{I,N}}{K_{I,N} + c_N} - \\ -\frac{1}{Y_{MEV/LAC}} \cdot q_{\max}^{MEV} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC}^{MEV} \cdot c_X} \cdot \frac{K_{I,N}^{MEV}}{K_{I,N}^{MEV} + c_N} \quad (2)$$

$$\sigma_N = -\frac{1}{Y_{X/N}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC} \cdot c_X} \cdot \frac{c_N}{c_N + K_N \cdot c_X} \quad (3)$$

$$\pi_{MEV} = q_{\max}^{MEV} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC}^{MEV} \cdot c_X} \cdot \frac{K_{I,N}^{MEV}}{K_{I,N}^{MEV} + c_N} + k_{MEV} \cdot c_{LAC} \quad (4)$$

$$\mu = \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC} \cdot c_X} \cdot \frac{c_N}{c_N + K_N \cdot c_X} \quad (5)$$

where: $Y_{X/LAC}$, $Y_{MEV/LAC}$ and $Y_{X/N}$ are yield coefficients, μ_{max} is maximum specific biomass growth rate, q_{max}^{MEV}, k_{MEV} are rate constants, K_N, K_{LAC}, K_{LAC}^{MEV} are affinity constants and $K_{I,N}^{MEV}$, $K_{I,N}$ are inhibition constants. More details concerning model parameters were presented in [31].

The model was tested for the batch cultivations in the wide range of the initial lactose from 5 to 40 g l^{-1} and organic nitrogen from 2 to 12 g l^{-1} concentrations and for the lactose-fed discontinuous fed-batch process. The exemplary model curves and experimental data are shown in Fig. 11. The list of parameter values can be found in [31].

Conclusions

Upon this review several conclusions can be drawn with regard to the relationships between the culturing conditions, fungal morphology and lovastatin production by *Aspergillus terreus*. Various kinetic relationships governing the described process are presented as well.

- The choice of the carbon source is the crucial factor for lovastatin biosynthesis by *A. terreus*. The majority of researchers agree that the use of a slowly degradable carbon sources such as lactose, starch or glycerol is the most favorable for this process.
- In order to produce lovastatin it is necessary to apply the complex organic nitrogen sources. A large variety of them can be used for this process. So can single amino acids and they are the simplest organic nitrogen source recommended. The use of ammonium ions or nitrates prevents *A. terreus* from lovastatin production. At the same time there is an agreement that elevated organic nitrogen levels inhibit lovastatin production.
- As far as the feeding with carbon source is concerned, the fed-batch culture is favorable for lovastatin production. The feeding solution should not contain organic nitrogen due to the above-mentioned inhibition.
- The supplementation of the cultivation media with such compounds as B-group vitamins, methionine or butyrolactone I in the amounts up to several tens of mg per liter facilitates lovastatin biosynthesis.
- There are controversies with regard to the effect of oxygen concentration on lovastatin biosynthesis. According to some authors, elevated oxygen

saturation is not favorable for lovastatin biosynthesis. Others suggest that the aeration of the broth with the gas enriched with oxygen increases lovastatin production.

- The influence of pH on lovastatin biosynthesis is generally not of the highest importance. One agrees that initial pH should be about 6.5. The profile of pH depends on the medium composition and the control of pH rather does not lead to the increase in lovastatin production.
- The formation of other polyketide metabolites by *A. terreus* in the lovastatin process has been underestimated in the research so far. The formation of the most profound by-product (+)-geodin is strongly carbon-dependent, thus facilitated in C-source-fed fed-batch cultivations, and is more sensitive to elevated organic nitrogen levels. Furthermore, high aeration rates of the broth increase (+)-geodin formation. The control of pH at the level slightly above neutral leads to the decrease of (+)-geodin production.
- Few correlations between the formation of polyketide metabolites by *A. terreus* and morphology of hyphae can be found. The smaller pellets (diameter lower than 2 mm) are the most favorable for lovastatin biosynthesis. No correlation between pellet size and (+)-geodin formation is found. Lovastatin formation is also connected with the metamorphosis of hyphae and its specific formation rate is linearly dependent on the amount of growing cells. With regard to (+)-geodin, its formation is not correlated with the presence of active cells.

- From the kinetic point of view lovastatin formation is mixed-growth associated. Its biosynthesis is extensive already in the trophophase but can be continued in the idiophase, if sufficient amount of carbon is present in the medium. Lovastatin biosynthesis may be delayed, if too much organic nitrogen is present in the medium. Nitrogen starvation is believed to be favorable for both lovastatin and (+)-geodin biosynthesis. As opposed to lovastatin, (+)-geodin formation is non-growth associated. This metabolite is excreted to the medium in the idiophase and this process is especially efficient, if high levels of carbon still remains in the medium.

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Figure legends

Fig. 1. Simplified scheme of lovastatin biosynthesis. Abbreviations used: SAM – **S**-**A**denosyl-L-**M**ethionine, LNKS – **L**ovastatin **N**ona**K**etide **S**ynthase, LDKS - **L**ovastatin **D**i**K**etide **S**ynthase, lovA, lovB, lovC, lovD, lovF – genes responsible for coding of the presented enzymes. For detailed information concerning this pathway including the nine-step formation of 4a,5-dihydromonacoline L and lovastatin biosynthesis gene cluster refer to [9,10].

Fig. 2. Simplified scheme of (+)-geodin biosynthesis upon [14]. The enzymes catalyzing the reactions: (0) uncharacterized octaketide synthase, (1) emodin anthrone oxygenase, (2) emodin O-methyltransferase (3), questin oxygenase, (4) desmethylsulochrin O-methyltransferase, (5) chloroperoxidase, (6) dihydrogeodin oxidase; the biochemical characterization of some enzymes can be found in [18-20]

Fig. 3. Lovastatin (LOV) biosynthesis at the varying initial lactose (LAC_0) concentration; initial yeast extract concentration equaled 4 g l^{-1} [31]

Fig. 4. Lovastatin (LOV) biosynthesis and organic nitrogen (N) utilization at the varying initial yeast extract (YE_0) concentration; initial lactose concentration equaled 20 g l^{-1} [31]

Fig. 5. (+)-geodin (GEOD) biosynthesis at the varying initial lactose (LAC_0) and yeast extract (YE_0) concentrations; initial yeast extract and lactose concentrations equaled 4 and 20 g l^{-1} respectively [35]

Fig. 6. Lovastatin and (+)-geodin biosynthesis in batch and lactose-fed discontinuous fed-batch shake flask culture; initial lactose and yeast extract

concentrations 20 and 4 g l^{-1}, lactose concentration in the feed equaled 100 g l^{-1} [35]

Fig. 7. Influence of the aeration rate on lovastatin and (+)-geodin biosynthesis in a stirred tank bioreactor; initial lactose and yeast extract concentrations equaled 20 g l^{-1} and 4 g l^{-1}, respectively [36]

Fig. 8. Influence of pH control and initial yeast extract concentration on lovastatin biosynthesis in the bioreactor; initial lactose concentration equaled 20 g l^{-1} [36]

Fig. 9. Influence of the initial number of spores in the preculture on pellet morphology and product formation (a) and differentiation of *A. terreus* hyphae (b); hyphal differentiation vs. product formation rate (c) in lovastatin and (+)-geodin biosynthesis; the cross section of *A. terreus* pellet (d): the external dark gray region of the sectioned pellet comprises the apical growing cells, the light gray radius-like structure of internal part consists of hyphal cells; based upon selected data from [47]

Fig. 10. Association of lovastatin and (+)-geodin with biomass growth at *A. terreus* determined upon the time changes of biomass (r_X), lovastatin (r_{LOV}) and (+)-geodin ($r_{GEOD)}$ volumetric formation rates (a) and the relationship between specific lovastatin (π_{LOV}) and (+)-geodin (π_{GEOD}) formation rates and specific biomass (μ) growth rate (b) [35]

Fig. 11. One of the fits of the model (eqs. from 2 to 5) to the experimental data; initial lactose and yeast extract concentration equaled 20 g l^{-1} and 4 g l^{-1}, respectively [31]

Table 1. Variety of applied carbon and nitrogen sources used for lovastatin production and their influence on this process

Carbon source	Nitrogen source	Product titer	Strain	Source
SUC (50 g l^{-1})	CSL (10 g l^{-1})	40% of the one in lactose medium	TUB F514	[27]
LAC (20 g l^{-1})	CSL (2.5 g l^{-1}) SM (2 g l^{-1})	400 mg LOV l^{-1}		
GLU (15 g l^{-1}) MLD (32 g l^{-1}) STR (20 g l^{-1}) LAC from milk (N/D)	CSL (5 g l^{-1}) YE (2.5 g l^{-1}) PM (25 g l^{-1})	1200 mg LOV l^{-1}	ATCC20541 mutant designated as DRCC122	[33]
GLU 45 g l^{-1}	Na-Glu (12.5 g l^{-1})	200 mg LOV l^{-1}	ATCC74135	[28]
LAC (53 g l^{-1})	PM (24 g l^{-1}) YE (2.5 g l^{-1})	120 mg LOV l^{-1}		
GLU (45 g l^{-1}) LAC from milk (11 g l^{-1})	PM (24 g l^{-1}) YE (2.5g l^{-1})	300 mg LOV l^{-1} 1.5 g EtOH l^{-1}		
GLU (45 g l^{-1})	ammonium tartrate, nitrates	no lovastatin		
FRU 20 g l^{-1} GLY (20.44 g l^{-1}) LAC (20 g l^{-1})	YE or SM or CSL (0.144-0.172 g N l^{-1})	40-120 mg LOV l^{-1}	ATCC20542	[30]
GLU (45 g l^{-1})	YE (12.5 g l^{-1})	12 mg LOV l^{-1} 10 g EtOH l^{-1} putative terpenes	ATCC20542	[29]
LAC (20 g l^{-1})	YE (8 g l^{-1})	35 mg LOV l^{-1}		
LAC (5-40 g l^{-1})	YE (2-12 g l^{-1})	5-110 mg LOV l^{-1}	ATCC20542	[31]

CSL – corn steep liquor, EtOH – ethanol, FRU fructose, GEOD – (+)-geodin, GLU –glucose, GLY – glycerol, LAC – lactose, LOV – lovastatin, MLD – maltodextrin, Na-Glu – sodium glutamate, PM – peptonized milk, SM – soybean meal, STR – starch, SUC – sucrose, TP – tomato paste, YE – yeast extract,

Table 2. Influence of the supplementing of the cultivation media with B-group vitamins on lovastatin and (+)-geodin production (yield coefficients and volumetric formation rates for lovastatin come from [39])

Supplementation	Amount added (mg l^{-1})	Yield coefficient			Maximum volumetric lovastatin formation rate (mg LOV l^{-1} h^{-1})	Maximum volumetric geodin formation rate (mg GEOD l^{-1} h^{-1})
		$Y_{LOV/LAC}$ (mg LOV g LAC^{-1})	$Y_{LOV/X}$ (mg LOV g X^{-1})	$Y_{GEOD/LAC}$ (mg GEOD g LAC^{-1})		
None	-	2.46±0.08	5.44±0.41	0.21±0.04	0.508	0.085
thiamine (B_1)	0.1	2.31±0.24	4.54±0.61	0.34±0.07	0.434	0.101
riboflavin (B_2)	0.066	2.34±0.25	4.29±0.66	0.34±0.06	0.650	0.064
Ca-pantothenate (B_5)	1	3.23±0.24	5.88±0.84	0.26±0.06	0.626	0.096
pyridoxine (B_6)	5	2.83±0.18	6.48±0.74	0.26±0.08	0.643	0.091
nicotinamide (PP)	6	2.45±0.21	4.79±0.79	0.22±0.05	0.621	0.073
mixture B_1/B_2/ B_5/B_6/ PP	0.04/0.664/ 0.664/0.664/ 5.31	0.496 ±0.038	18.31±1.94	-	0.637	-

Table 3. Influence of oxygen on lovastatin and (+)-geodin biosynthesis

Nutrients	Aeration, vvm	Oxygen saturation	Product titer	Strain	Source
GLU 100 g l^{-1} CSL 20 g l^{-1} TP 5 g l^{-1} beer yeasts 20 g l^{-1}	0.7-1.5	35%	25 U l^{-1} (LOV)*	ATCC20541	[37]
		70%	160 U l^{-1} (LOV)*		
		80%	125 U l^{-1} (LOV)*		
LAC 70 g l^{-1} CSL 5 g l^{-1} YE 5 g l^{-1} SM 5 g l^{-1}	no oxygen vector	shake flask	260 mg LOV l^{-1}	ATCC20542	[43]
		20%	450 mg LOV l^{-1}		
	2.5% dodecane	shake flask	500 mg LOV l^{-1}		
		70%	80 mg LOV l^{-1}		
LAC 8-48 g C l^{-1} SM 0.2-0.6 g N l^{-1}	20%**	not given	47.2-121.1 mg LOV l^{-1}	ATCC20542	[40]
	50%**		61.9-188.6 mg LOV l^{-1}		
	80%**		104.8-202.8 mg LOV l^{-1}		
LAC (114.26 g l^{-1}) SM (0.15-0.92 g N l^{-1})	0.5 80%**	not given	25 mg LOV l^{-1}	ATCC20542	[41]
	1.0 20%**		10-50 mg LOV l^{-1}		
	80%**		50-300 mg LOV l^{-1}		
	1.5 80%**		10-100 mg LOV l^{-1}		
LAC (20 g l^{-1}) YE (2 or 4 g l^{-1})	0.308	8-40%***	38-72 mg LOV l^{-1} 4-58 mg GEOD l^{-1}	ATCC20542	[36]
	0.513	25-55%***	12 mg LOV l^{-1} 67 mg GEOD l^{-1}		
	1.026	40-80%***	<10 mg LOV l^{-1} 120 mg GEOD l^{-1}		

CSL – corn steep liquor, EtOH – ethanol, FRU fructose, GEOD – (+)-geodin, GLU –glucose, GLY – glycerol, LAC – lactose, LOV – lovastatin, MLD – maltodextrin, Na-Glu – sodium glutamate, PM – peptonized milk, SM – soybean meal, STR – starch, SUC – sucrose, TP – tomato paste, YE – yeast extract,

* direct lovastatin concentration not given

** amount of oxygen (v/v) in the aeration gas

*** uncontrolled oxygen saturation

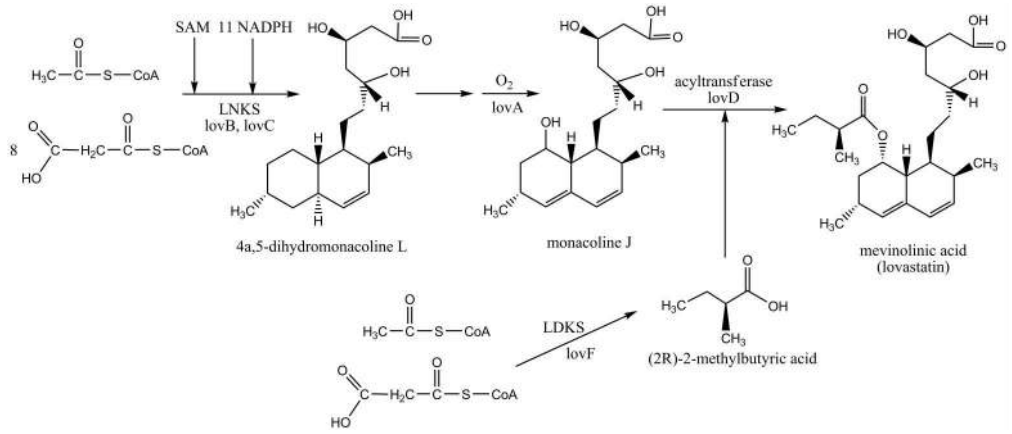

Fig.1 Simplified scheme of lovastatin biosynthesis. Abbreviations used: SAM – S-Adenosyl-L-Methionine, LNKS – Lovastatin NonaKetide Synthase, LDKS - Lovastatin DiKetide Synthase, lovA, lovB, lovC, lovD, lovF – genes responsible for coding of the presented enzymes. For the detailed information concerning this pathway including the nine-step formation of 4a,5-dihydromonacoline L and lovastatin biosynthesis gene cluster refer to [9,10].
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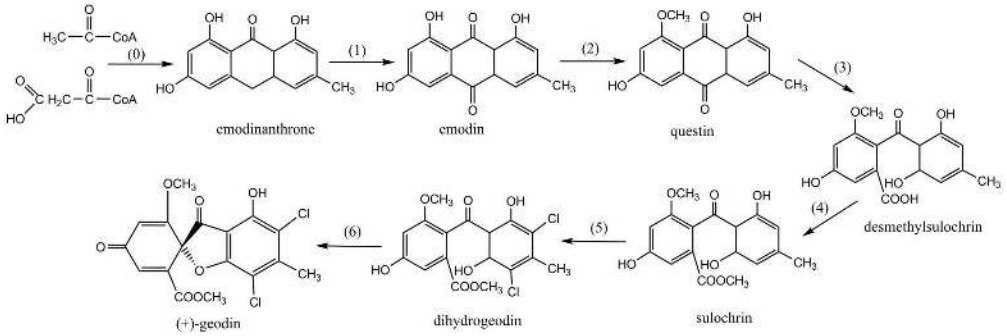

Fig.2. Simplified scheme of (+)-geodin biosynthesis upon [14]. The enzymes catalyzing the reactions: (0) uncharacterized octaketide synthase, (1) emodin anthrone oxygenase, (2) emodin O-methyltransferase (3), questin oxygenase, (4) desmethylsulochrin O-methyltransferase, (5) chloroperoxidase, (6) dihydrogeodin oxidase; the biochemical characterization of some enzymes can be found in [18-20]
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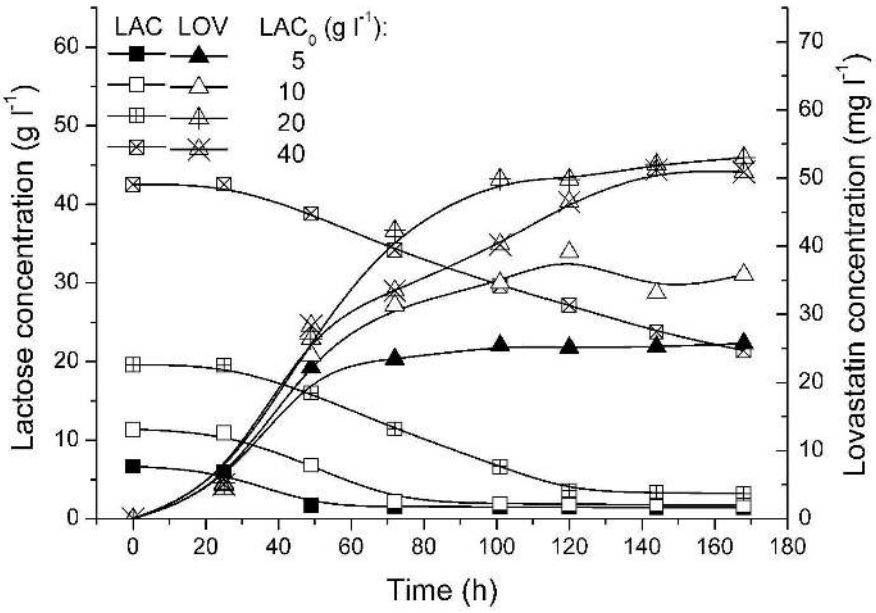

Fig.3. Lovastatin (LOV) biosynthesis at the varying initial lactose (LAC_0) concentration; initial yeast extract concentration set at 4 g l^{-1} [31]
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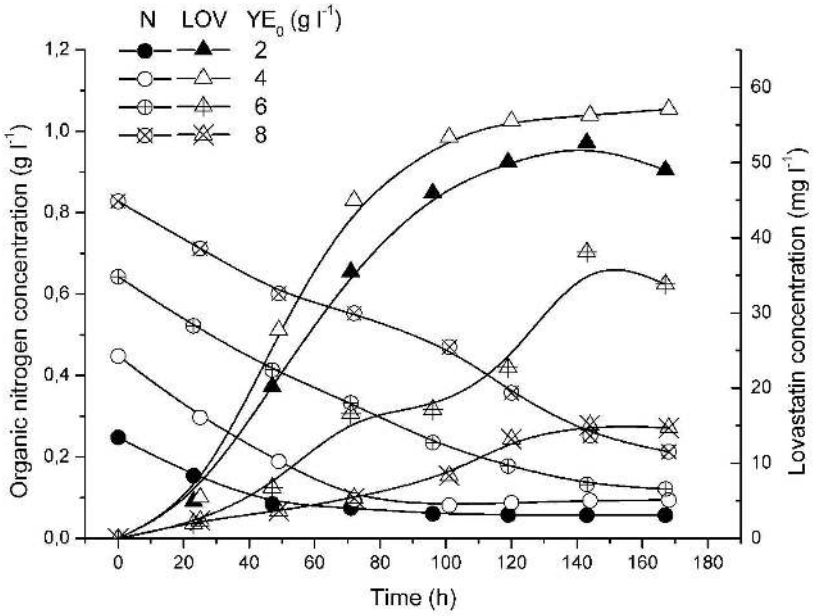

Fig.4 Lovastatin (LOV) biosynthesis and organic nitrogen (N) utilization at the varying initial yeast extract (YE_0) concentration; initial lactose concentration set at 20 g l^{-1} [31]
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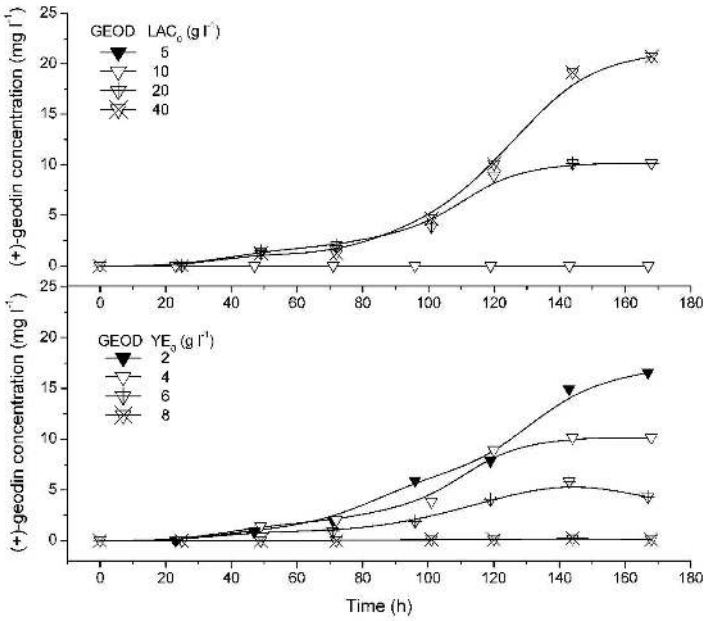

Fig.5 (+)-geodin (GEOD) biosynthesis at the varying initial lactose (LAC_0) and yeast extract (YE_0) concentrations; initial yeast extract and lactose concentrations equal to 4 and 20 g l^{-1} respectively [35]
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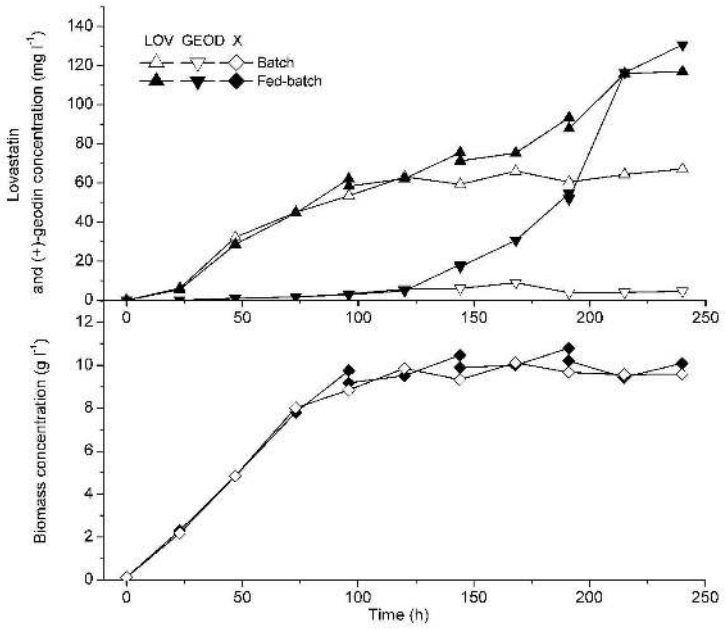

Fig.6 Lovastatin and (+)-geodin biosynthesis in batch and lactose-fed discontinuous fed-batch shake flask culture; initial lactose and yeast extract concentrations 20 and 4 g l^{-1}, lactose concentration in the feed equaled 100 g l^{-1} [35]
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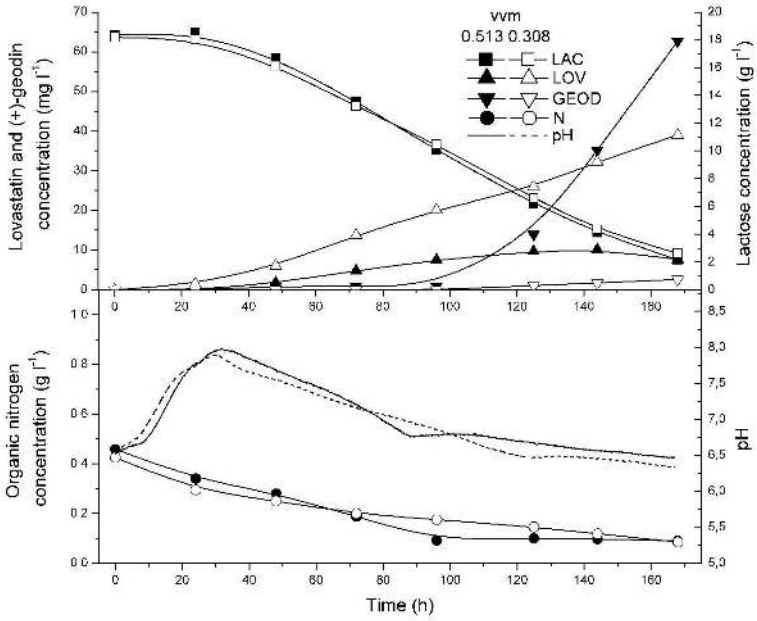

Fig.7 Influence of the aeration rate on lovastatin and (+)-geodin biosynthesis in a stirred tank bioreactor; initial lactose and yeast extract concentration 20 g l^{-1} and 4 g l^{-1} [36]
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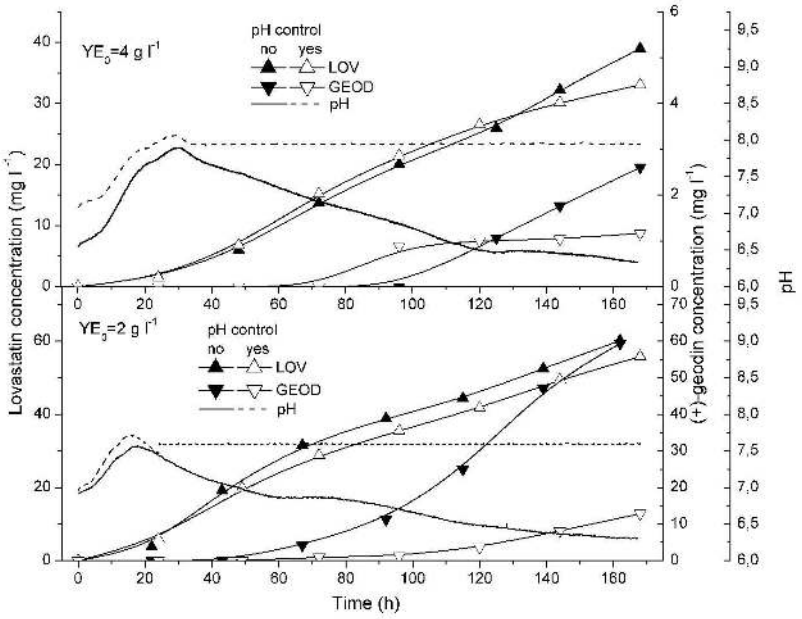

Fig.8 Influence of pH control and initial yeast extract concentration on lovastatin biosynthesis in the bioreactor; initial lactose concentration 20 g l^{-1} [36]
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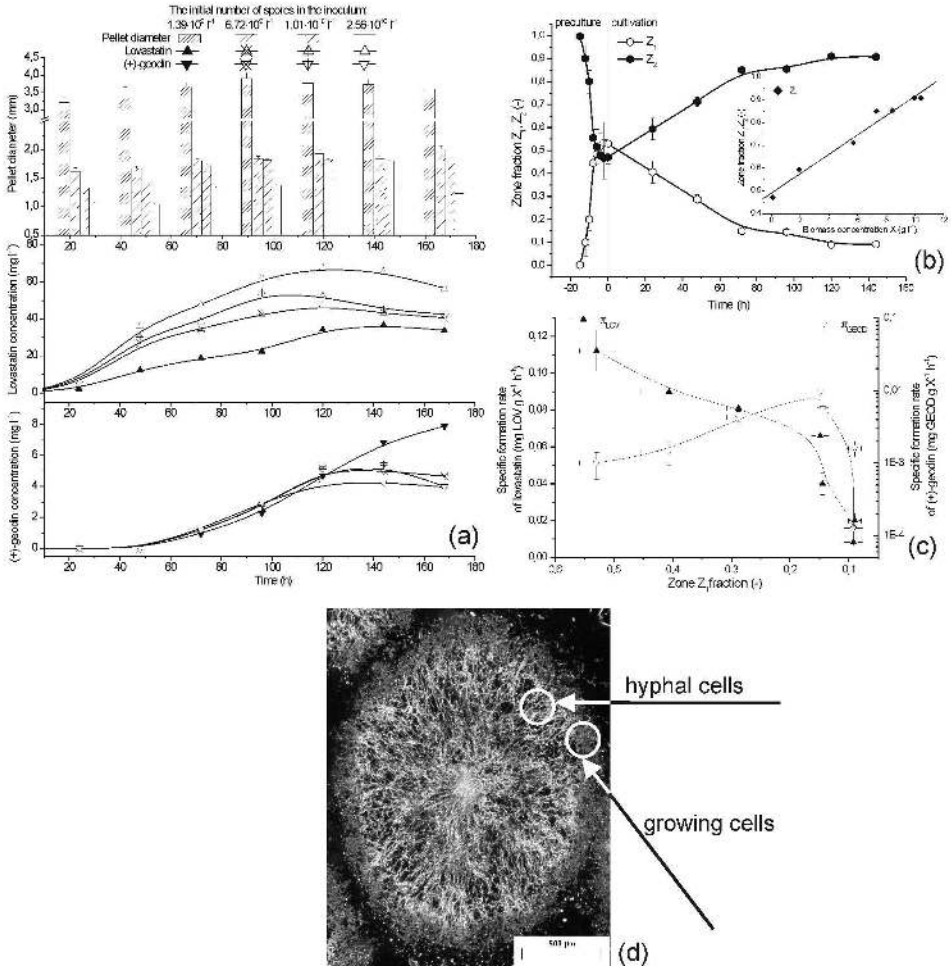

Fig. 9 Influence of the initial number of spores in the preculture on pellet morphology and product formation (a) and differentiation of A. terreus hyphae (b); hyphal differentiation vs. product formation rate (c) in lovastatin and (+)-geodin biosynthesis; the cross section of A. terreus pellet (d): the external dark gray region of the sectioned pellet comprises the apical growing cells, the light gray radius-like structure of internal part consists of hyphal cells; based upon selected data from [47]
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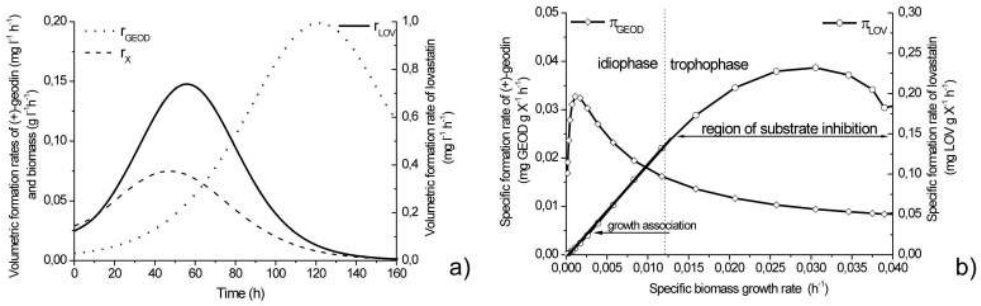

Fig.10 Association of lovastatin and (+)-geodin with biomass growth at A. terreus determined upon the time changes of biomass (rX¬), lovastatin (rLOV) and (+)-geodin (rGEOD) volumetric formation rates (a) and the relationship between specific lovastatin (πLOV) and (+)-geodin (πGEOD) formation rates and specific biomass (μ) growth rate (b) [35]
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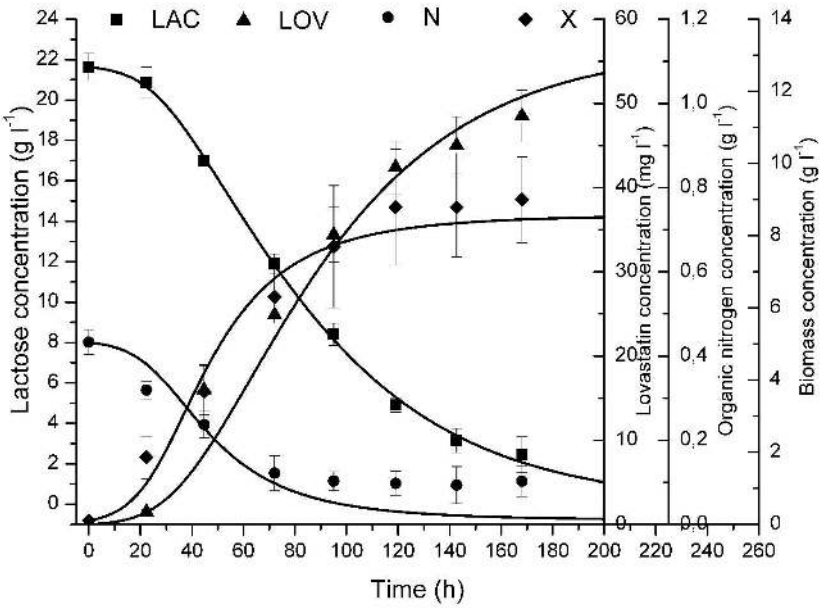

Fig.11 One of the fits of the model (eqs. from 2 to 5) to the experimental data; initial lactose concentration 20 g l^{-1}, initial yeast extract concentration 4 g l^{-1} [31]
286x201mm (600 x 600 DPI)