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Marcin Bizukojc, Stanislaw Ledakowicz

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

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6 **Review** ((11234 words))
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9 **Physiological, morphological and kinetic aspects of**
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11 **lovastatin biosynthesis by *Aspergillus terreus***
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15 Marcin Bizukojc*, Stanislaw Ledakowicz

16
17 Department of Bioprocess Engineering, Technical University of Lodz, ul.
18
19 Wolczanska 213, 90-924 Lodz, Poland
20
21

22 * Author for correspondence (fax: +48 42 631 56 63, e-mail:
23
24 marcin.bizukojc@p.lodz.pl)
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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].

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6 Lovastatin is the international name of the substance, which is actually the
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8 lactone form of the natural β -hydroxy acid, called mevinolinic acid. Mevinolinic
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10 acid is the active compound in the human organism and is also excreted from the
11
12 fungal cells into cultivation media. Its systematic name is 2-methyl-1,2,3,7,8,8a-
13
14 hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-piran-2-yl)ethyl]-
15
16 1-naphtalenylesther of butanoic acid. Its lactone, i.e. lovastatin, is formed in the
17
18 course of the extraction of mevinolinic acid from cultivation media. The lactone
19
20 becomes the active β -hydroxy acid again in the human alimentary canal. Although
21
22 everything, which is going to be written further in this review, refers actually to
23
24 mevinolinic acid as the product of *A. terreus* metabolism, for the sake of
25
26 simplicity the name “lovastatin” will be used as more authors prefer it.
27
28 Nevertheless, it is worth mentioning that in the earlier works other names for the
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30 lactone form of this compound are used, e.g. mevinolin [4] or monacoline K [2] or
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32 MSD803 [5].
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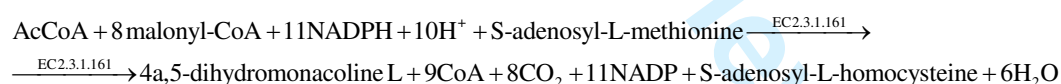
38
39 **From a microbial metabolism point of view**, lovastatin belongs to the large
40
41 group of secondary metabolites called polyketides. Biosynthesis of such
42
43 metabolites usually consists of at least two stages. The first stage catalyzed by a
44
45 polyketide synthase (PKS) is always obligatory. There are various types of
46
47 polyketide synthases, which have both different structures and functions. **Not to**
48
49 **focus on details**, PKSs can be divided into iterative and module synthases. The
50
51 iterative PKS is a single enzyme containing between five **and** seven active sites of
52
53 various catalytic activities **such** as ketoreduction, enoilreduction, dehydration,
54
55 methyl group transfer, acyl group transfer, cyclization and aromatization. These
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6 active sites can be used **repeatedly** in a single reaction to form an individual
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8 product molecule. The module PKS is an enzymatic complex, which fulfills the
9
10 same functions, as the one mentioned above, but each active site of the enzyme is
11
12 used only once in the formation of an individual product molecule. The detailed
13
14 description of different types of PKSs can be found in [6] and [7].

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16
17 It frequently **occurs** that a PKS product is further subjected to other
18
19 enzymatic reactions. These reactions are called post-PKS tailoring steps and they
20
21 are usually oxidations, esterifications or methylations. The detailed description of
22
23 the post-PKS tailoring was presented in [8].
24
25

26
27 Lovastatin biosynthesis by *A. terreus* is performed in two stages. The first
28
29 stage is catalyzed by lovastatin nonaketide synthase (LNKS, EC 2.3.1.161), which
30
31 belongs to type I polyketide synthases and catalyzes the iterative nine-step
32
33 formation of the polyketide [9-12].
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37 This PKS stage leads to 4a,5-dihydromonacoline L and the stoichiometric
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39 equation can be written as:
40



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45 The post-PKS tailoring hydroxylation and oxidation steps are performed with the
46
47 participation of molecular oxygen. The obtained intermediate monacoline J is then
48
49 transesterified with (2R)-2-methylbutyric acid, which is another intermediate
50
51 formed in the reaction catalyzed by lovastatin diketide synthase (LDKS). The
52
53 simplified scheme of lovastatin biosynthesis is presented in Fig.1. The mechanism
54
55 of lovastatin biosynthesis is quite well recognized, including the gene cluster with
56
57 Open Read Frames (ORFs) coding the sequences of LNKS, LDKS, oxidizing and
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6 transestrifying enzymes. Also the functions of other ORFs, which play important
7
8 roles in lovastatin biosynthesis, were recognized. Additionally, the amino acid
9
10 sequences of the enzymes engaged in biosynthesis of lovastatin are known [9].
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12 For more details concerning the biochemical and genetic aspects of lovastatin
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14 biosynthesis papers by Kennedy et al. and Sunderland et al. can be recommended
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16 [9,10].
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19
20 Although lovastatin is the most important metabolite of *A. terreus*, this
21
22 microorganism of a rich secondary metabolism is also capable of biosynthesizing
23
24 other compounds. It was already reported in the thirties that *A. terreus* strains
25
26 were capable of formation of a substance, which was then called (+)-geodin [13].
27
28 Its systematic name is (+)-5,7-dichloro-4-hydroxy-6'-methoxy-6-methyl-3,4'-
29
30 dioxomethylester of spiro(benzofuran-2(3H),1'-(2,5)-cyclohexadien)-2'-carboxylic
31
32 acid and it is also a product of the polyketide pathway and, as lovastatin, is
33
34 formed from malonyl-CoA and acetyl-CoA [14].
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39 The metabolic pathway leading to (+)-geodin formation differs greatly
40
41 from the lovastatin one. First of all, the PKS stage comprises the action of an
42
43 uncharacterized octaketide synthase. Next, there are more post-PKS tailoring
44
45 steps, which lead to the formation of such compounds as emodin, questin,
46
47 sulochrin and finally (+)-geodin (Fig. 2). Other compounds as asterric acid or
48
49 methyl asterrate might be formed from (+)-geodin and sulochrin [15,16].
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54 Recent papers have reported on (+)-geodin. Askenazi et al. investigated the
55
56 transcriptional and metabolite profiles of the lovastatin-producing fungi and they
57
58 found significant amounts of (+)-geodin in several *Aspergilli* strains, including
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6 lovastatin producer *A. terreus*. They also managed to disrupt (+)-geodin
7
8 biosynthesis with the use of genetic engineering techniques [14].
9

10 Hargreaves et al. managed to isolate and determine the structure of (+)-
11 geodin and several derivatives of asterric acid from *Aspergillus sp.* They also
12 showed their antifungal properties [16]. Also Couch and Gaucher mentioned (+)-
13 geodin in the context of the elimination of sulochrin biosynthesis [17]. Several
14 enzymes of (+)-geodin biosynthetic pathway were also isolated and characterized
15 [18-20].
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24 The question whether (+)-geodin is only a by-product in lovastatin
25 biosynthesis remains unanswered, as Askenazi et al. sustained that it is the
26 intermediate in the biosynthesis of other natural products [14]. What is more,
27 several scientists announced that (+)-geodin showed the activity as a glucose
28 uptake stimulator towards rat adipocytes and enhanced the fibrinolytic activity of
29 vascular endothelial cells [21,22].
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38 Sulochrin [methyl 2-(2,6-dihydroxy-4-methylbenzoil)-5-hydroxy-3-
39 metoxybenzoate], another octaketide metabolite (Fig. 2) is an antibiotic [23] and
40 was mentioned as the co-metabolite in lovastatin biosynthesis by Schimmel et al.
41 [24]. It is also thought to be an undesired metabolite by Couch and Gaucher, who
42 obtained lovastatin-producing *A. terreus* mutants incapable of forming this
43 metabolite [17]. Another important but non-polyketide metabolite of *A. terreus* is
44 itaconic acid, which is biosynthesized by various *A. terreus* strains. Nevertheless,
45 it is rarely mentioned in the context of lovastatin biosynthesis by the basic
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6 lovastatin-producing strain ATCC20542. Only Lai et al. claimed that they found
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8 this metabolite, while using either lactose or glucose based media [25].
9

10 11 12 **Physiological approach to investigate lovastatin biosynthesis**

13 *Influence of carbon and nitrogen sources*

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18 The choice of the relevant carbon and nitrogen sources for lovastatin
19
20 biosynthesis has remained somewhat controversial since the very beginning of
21
22 lovastatin history, when Monaghan et al. patented **its biosynthesis process** [5]. The
23
24 research on lovastatin biosynthesis by *A. terreus* was performed with the use of
25
26 diverse nutrients and even now it would be uncertain to claim that there is an
27
28 established medium composition for this process, even with regard to carbon and
29
30 nitrogen source.
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34 The diversity of nutrients for lovastatin biosynthesis can be noticed in the
35
36 patent by Monaghan et al., who proposed glucose, oat meal, corn steep liquor,
37
38 tomato paste, starch **or** yeasts in the seed culture and lactose, yeast extract, malt
39
40 extract or dextrose in the cultivation medium [5]. The early publications did not
41
42 focus on the aspects of the influence of nutrients on lovastatin biosynthesis [4,26].
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46 Reviewing the influence of nutrients on lovastatin biosynthesis, it is worth
47
48 **mentioning** with the work of Szakacs et al., who tested lactose with corn steep
49
50 liquor and sucrose with corn steep liquor for the Hungarian strain *A. terreus* TUB
51
52 F-514 [27]. **It was observed** that the highest lovastatin titer of 400 mg l⁻¹ was
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54 obtained with the use of lactose as the carbon source. Lovastatin titers on sucrose
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56 were **40% of that on lactose**.
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6 One of the most detailed experiments concerning media composition for
7
8 lovastatin production was performed by Hajjaj et al. [28]. They tested **the growth**
9
10 **of** ATCC74135 strain on synthetic and complex media. Glucose and sodium
11
12 glutamate were applied in the synthetic media, whereas lactose or glucose,
13
14 peptonized milk (also lactose source) and yeast extract in the complex media. In
15
16 the synthetic media, fast glucose utilization was observed from the level of 45 g l^{-1}
17
18 down to zero within 50 hours with the specific glucose uptake rate of 0.196 g g^{-1}
19
20 h^{-1} . Lactose was then far slower assimilated and even not ultimately exhausted:
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22 from 50 g l^{-1} to about half of this concentration within 300 hours. Nevertheless,
23
24 when glucose was being assimilated, a side effect was observed. Ethanol was
25
26 excreted, up to 1.3 g l^{-1} in the phase of fast glucose uptake. Later on, this ethanol
27
28 was reutilized. The same phenomenon was observed by Bizukojc and Ledakowicz
29
30 in the experiments with ATCC20542 strain [29]. **Glucose and yeast extract were**
31
32 **used** at concentrations of 45 g l^{-1} and 12.5 g l^{-1} , respectively. The maximum
33
34 ethanol concentration reached 10 g l^{-1} **at glucose depletion**. Although ethanol was
35
36 further utilized, as it had been observed by Hajjaj et al. [28], it did not contribute
37
38 to lovastatin formation. **Lovastatin** concentration did not increase after glucose
39
40 depletion. Additionally, Bizukojc and Ledakowicz claimed that unidentified
41
42 fruity-smelling compounds, probably terpenes, were simultaneously produced.
43
44 When glucose was replaced with lactose at **an** initial concentration of 20 g l^{-1}
45
46 neither ethanol nor other fragrant compounds were formed [29]. These data
47
48 suggest that slowly degradable carbon sources such as lactose are favorable for
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50 lovastatin production. It is also worth mentioning that Hajjaj et al. **obtained good**
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6 lovastatin yields with the use of a glucose and lactose mixture in the experiments,
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8 in which they applied peptonized milk (the source of lactose) as the nitrogen
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10 source with glucose as the carbon source (Table 1) [28].
11

12
13 The hypothesis that slowly degradable carbon sources are preferred in
14
15 lovastatin production is also claimed by Casas Lopez et al. [30]. They used the
16
17 basic strain ATCC20542 and tested fructose, glycerol and lactose as the carbon
18
19 sources. These authors never used glucose. They believe that it is the invalid
20
21 carbon source for lovastatin biosynthesis by *A. terreus* as it causes catabolic
22
23 repression. Analyzing substrate uptake rate, Casas Lopez et al. found that,
24
25 irrespective of the nitrogen source used, fructose was the fastest utilized carbon
26
27 source, followed by glycerol and lactose [30]. Apart from the proper choice of
28
29 carbon and nitrogen source they drew an attention to another issue, i.e. carbon to
30
31 nitrogen (C/N) ratio. The increase of C/N ratio in the idiophase even up to 41.3,
32
33 which was achieved by the feeding of the culture with the carbon source, led to an
34
35 elevated lovastatin yield (Table 1) [30].
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41 The systematic approach in testing the influence of the initial carbon
42
43 source concentration on lovastatin biosynthesis was performed by Bizukojc and
44
45 Ledakowicz [31]. In this work lactose was used as the carbon source and yeast
46
47 extract as the nitrogen source. It was shown that lovastatin biosynthesis was
48
49 strongly limited by lactose (Fig. 3). At the initial lactose concentrations of 5 and
50
51 10 g l⁻¹, lovastatin formation ceased as a result of lactose depletion. However, the
52
53 increase in the initial lactose concentration from 20 to 40 g l⁻¹ did not result in any
54
55 significant increase in lovastatin titer. Furthermore, lactose was not exhausted at
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6 its higher initial concentration of 40 g l⁻¹ [31]. Similar results were found by
7
8 Hajjaj et al. [28]. Upon these observations it can be assumed that *A. terreus*
9
10 cultivation in the carbon source fed fed-batch culture would prove the efficient
11
12 method to perform lovastatin production. This issue shall be discussed further.
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16 One of the most important issues connected with any microbial cultivation
17
18 is to test, if there is a chance to apply the mineral nitrogen sources. Complex
19
20 nitrogen sources are not welcome in the industrial processes as their chemical
21
22 composition is not always reproducible and they are at times expensive. Hajjaj et
23
24 al. investigated this aspect of lovastatin biosynthesis in detail, testing inorganic
25
26 nitrogen sources and amino acids [28].
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29
30 Generally, amino acids are utilized by fungi only as the nitrogen source
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32 because no ammonium ions are found in the broth. *A. terreus* responds similarly
33
34 and the optimum amino acids to be utilized by this fungus for lovastatin
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36 biosynthesis was either sodium glutamate or histidine. Several tens of mg
37
38 lovastatin per liter was biosynthesized, if these amino acids were applied [28]. On
39
40 the other hand, Hajjaj et al. found that *A. terreus* could utilize inorganic and
41
42 organic ammonium salts as ammonium tartrate or nitrates for biomass growth.
43
44 Unfortunately, in such media no lovastatin biosynthesis was observed because
45
46 such nitrogen sources, when being assimilated, acidify the broth [28].
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49 Furthermore, Lai et al. mentioned that ammonium sulfate was not suitable for
50
51 lovastatin production in their experiments performed to obtain data for the
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53 statistical medium design [32]. Therefore, even if synthetic media were to be used
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6 for lovastatin production, at least one amino acid as a nitrogen source should be
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8 present.

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10 The complex nitrogen source for lovastatin biosynthesis by *A. terreus* has
11 a wide selection: yeast extract, corn steep liquor, soybean meal, oat meal,
12 peptonized milk are the most frequently used [inter alia: 28,30,31,33,34]. Due to
13 the fact that these are mixtures of various organic nitrogen compounds, mainly
14 amino acids, the detailed investigation on their influence on lovastatin
15 biosynthesis can only be quantified using organic nitrogen concentration as the
16 analytical parameter.
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27 Casas Lopez et al. noticed that lovastatin biosynthesis took place in the
28 nitrogen-limited phase during cultivation and that starvation, with regard to
29 organic nitrogen, induced *A. terreus* to produce lovastatin [30]. Within contrast
30 Hajjaj et al. claimed that lovastatin formation was observed only when lactose
31 was depleted. It was concluded that lactose starvation was favorable for lovastatin
32 biosynthesis [28]. Bizukojc and Ledakowicz, having systematically tested the
33 influence of both lactose and yeast extract on lovastatin production, found that
34 lactose consumption was unquestionably required for lovastatin production (Fig.
35 4), starting in biomass growth phase and continuing into the idiophase [31]. It was
36 the organic nitrogen, which inhibited lovastatin biosynthesis in batch processes
37 and its initial level should not be too high (Fig. 4).
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53 To sum up the issues concerning the influence of C- and N-sources on
54 lovastatin production by *A. terreus*, various approaches to the formulation of
55 medium composition and resulting lovastatin titers were collected in Table 1.
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6 The aforementioned considerations focused only on the effect of the media
7 composition on lovastatin formation. But lovastatin is not the sole metabolite
8 produced by *A. terreus*. As it was mentioned in the introduction, some authors
9 have referred to other metabolites, as (+)-geodin, produced by this fungus [14-16]
10 and only Bizukojc and Ledakowicz tested the influence of media composition and
11 process conditions on simultaneous lovastatin and (+)-geodin biosynthesis
12 [35,36].
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22 According to Bizukojc and Ledakowicz (+)-geodin biosynthesis is more
23 dependent on the initial lactose concentration than lovastatin [35,36]. They
24 observed it only at higher initial lactose concentrations of 20 and 40 g l⁻¹ (Fig 5)
25 and the presence of lactose in the late idiophase in the broth was necessary to start
26 and prolong its formation. Similar results were obtained in fed-batch cultivations
27 (Fig. 6). The depletion of lactose eventually ceased (+)-geodin formation.
28 Furthermore, (+)-geodin biosynthesis was also more sensitive towards organic
29 nitrogen concentration as the inhibitive effect was in this case stronger (Fig. 5).
30 Under nitrogen starvation conditions (+)-geodin biosynthesis was the most
31 efficient [35,36].
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46 The issue of establishing feeding strategies in fed-batch cultivations is also
47 closely connected with the choice of carbon and nitrogen sources and their
48 concentrations. Some authors proved that carbon source feeding was favorable for
49 lovastatin biosynthesis. Novak et al. used repeated fed-batch culture with glucose
50 as the carbon source. As the depletion of glucose ceased lovastatin formation, they
51 fed the culture twice with glucose solution, achieving elevated lovastatin yield by
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6 about 37%. They also noticed that if the feeding was repeated more times, it did
7
8 not assure **increased** lovastatin productivity [37].
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11 The strategy applied by Sitaram Kumar et al. was different as there were
12
13 different carbon sources in the batch medium and in the feed. The batch medium
14
15 contained glucose, maltodextrin and starch, while the feed contained only
16
17 maltodextrin. Their culture was fed from 72 hour of the run in 5 hour intervals.
18
19 Interestingly **the nitrogen source, in the form of** corn steep liquor was also fed into
20
21 the bioreactor but in **a** less frequent manner, every 24 hours. The latter might be
22
23 controversial as nitrogen is believed to inhibit lovastatin formation. Nevertheless,
24
25 lovastatin volumetric productivity was almost doubled due to this **feeding strategy**
26
27 [33].
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31
32 Bizukojc and Ledakowicz tested the effect of lactose feeding in the
33
34 discontinuous fed-batch culture on lovastatin and (+)-geodin formation by *A.*
35
36 *terreus* (Fig. 6). **It was** concluded that lactose feeding increased lovastatin titer
37
38 [31,35]. Furthermore, **it was** observed that (+)-geodin production was **increased**
39
40 **significantly** by lactose feeding, in both shake flask and bioreactor cultivations.
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42 **This was attributed to an elevated lactose concentration in the late idiophase**
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44 **[35,36].**
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49 The most detailed research on feeding strategies in lovastatin biosynthesis
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51 was performed by Rodriguez Porcel et al. They tested the following feeding
52
53 strategies: **either complete medium, medium without organic nitrogen or only**
54
55 **minerals without carbon and nitrogen.** Their processes were not typical fed-batch
56
57 **cultivations** but rather semi-continuous runs because biomass-free medium was
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6 retrieved from the bioreactor **during** the feeding phase. The nitrogen free medium
7
8 was **observed** to be the optimum **feed**, which increased lovastatin production by
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10 315% [38].
11

12 13 14 15 *Influence of other compounds on lovastatin biosynthesis* 16

17
18 Apart from **the** main nutrients, some authors have focused on the
19
20 supplementation of *A. terreus* cultivation media with special compounds added at
21
22 low concentrations from several to tens **of** mg per liter. The main motivation for
23
24 **this** approach is connected with the biochemical mechanisms **governing** lovastatin
25
26 biosynthesis, to be more precise the action of lovastatin nonaketide synthase (see
27
28 the stoichiometric equation). For example, precursors of NADP or CoA might
29
30 have had an impact on the process. A similar action can be expected in relation to
31
32 methionine, which is a component of the methyl group donor S-adenosyl-L-
33
34 methionine, in the third step of LNKS action [9].
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39 The effect of methionine supplementation was tested by Lai et al. They
40
41 claimed that when DL-methionine (100 mg l^{-1}) was added **at** 72 hour of
42
43 cultivation, lovastatin formation increased by 20%. **In contrast** L-methionine did
44
45 not exert such an effect [32]. They proposed two hypotheses to explain this:
46
47 **either D-methionine also plays a** role in the transfer of methyl groups or the
48
49 racemate activates the polyketide synthase. It is controversial **hypothesis**, as
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51 Kyoto Encyclopedia of Genes and Genomes (KEGG) does not indicate any
52
53 function of D-methionine in the metabolic maps. Nevertheless, L-methionine
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6 racemase, whose cofactor is pyridoxal-5'-phosphate, is mentioned both in KEGG
7
8 and Braunschweig Enzyme Database (BRENDA) as EC 5.1.1.2.
9

10 Bizukojc et al. tested the effect of B-group vitamins supplementation on
11 lovastatin biosynthesis. Not only precursors of NADP (nicotinamide) and CoA
12 (calcium pantothenate), but also thiamine, riboflavin and pyridoxine were taken
13 into account. All these vitamins, excluding thiamine, increased the lovastatin
14 maximum volumetric formation rate. Calcium pantothenate increased both
15 lovastatin on lactose ($Y_{LOV/LAC}$) and lovastatin on biomass ($Y_{LOV/X}$) yield
16 coefficients and so did pyridoxine (Table 2) [39]. Bizukojc et al. also observed
17 that lactose utilization was decreased during the middle and late idiophase when
18 B-group vitamins were present in the medium. Because lovastatin biosynthesis is
19 lactose-dependent, as it was mentioned above, reduced lactose utilization and
20 resulting elevated lactose concentration contributed to increased lovastatin
21 formation. These results were further enhanced with the supplementation of the
22 vitamin mixture. It increased volumetric and specific lovastatin formation rates, as
23 well as yield coefficients $Y_{LOV/LAC}$ and $Y_{LOV/X}$ (Table 2) [39]. In terms of (+)-
24 geodin production, results were different. Both nicotinamide or riboflavin did not
25 increase the maximum (+)-geodin formation rate. However (+)-geodin on lactose
26 yield coefficients ($Y_{GEOD/LAC}$) were higher in all cases with vitamin
27 supplementation. During this study (+)-geodin on biomass yield coefficients
28 ($Y_{GEOD/X}$) were not calculated as they have no biological sense, if the definition of
29 yield coefficient is taken into account (Table 2). (+)-geodin formation was
30 determined to be non-growth associated. This will be discussed further.
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6 Another supplementary medium component was proposed by Schimmel et
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8 al. [24]. They cultivated *A. terreus* ATCC20542 on glucose, lactose, yeast extract
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10 and peptonized milk and in addition applied butyrolactone I [α -oxo- β -(*p*-
11 hydroxylphenyl)- γ -(*p*-hydroxy-*m*-3,3-dimethylallyl-benzyl)- γ -methoxycarbol- γ -
12 butyrolactone]. Butyrolactone I is a secondary metabolite produced by *A. terreus*.
13
14 The motivation for the use of the surplus dose of this metabolite was that this
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16 compound is known to act as a self-regulating factor in some bacteria. With
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18 respect to *A. terreus*, it, first of all, had an impact on the morphology of the
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20 fungus, increasing the amount of the branched hyphae, when added at 8 hour of
21
22 the run. Secondly, it increased the number of spores occurring in the submerged
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24 sporulation. Finally, lovastatin yield was almost tripled (up to 940 mg l⁻¹), if
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26 butyrolactone I was added at 100 μ M at 120 hour of the run. Nevertheless,
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28 sulochrin formation was also higher by about 82%. The best results were
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30 obtained, if butyrolactone I was added in the early idiophase, at the completion of
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32 the exponential phase [24].
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44 ***Influence of aeration rate and oxygen concentration on lovastatin biosynthesis***

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46 The aeration rate is one of the key factors influencing filamentous fungi
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48 growth and there are several papers, which focus on this aspect of lovastatin
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50 biosynthesis by *A. terreus*. Nevertheless, it is difficult to find the unanimous
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52 statements concerning the influence of aeration rate and oxygen saturation of the
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54 medium on lovastatin biosynthesis as different approaches to test the effects of
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56 oxygenation were used. Also, most of the information comes from three teams,
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6 who obtained, to a certain extent, contradictory results, probably due to different
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8 experimental procedures applied.
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10 The interesting idea to test the influence of oxygen on lovastatin
11 biosynthesis was proposed by Casas Lopez et al. [40]. Their paper concerned the
12 optimization of medium composition for lovastatin production. It is known that
13 for tens experiments to perform, while using response surface methodology or
14 other statistical optimization methods, hardly can the influence of oxygen be
15 tested due to the fact that such experiments are made in shake flasks not in
16 bioreactors. Casas Lopez et al. solved this problem using a controlled atmosphere
17 chamber with the aeration gas mixtures containing 20, 50 and 80% (v/v) oxygen,
18 respectively, for the cultivation of ATCC20542 strain. They found that the
19 aeration gas enriched with oxygen strongly increased lovastatin formation. These
20 preliminary results were later confirmed by the same researchers, who found the
21 positive effect of high aeration rate of the medium on lovastatin biosynthesis in
22 the bubble column [41]. They worked at three vvm values 0.5, 1.0 and 1.5 $l_{\text{air}} l^{-1}$
23 min^{-1} and the gas phase was either air or air enriched with oxygen at the level of
24 80% (v/v). At vvm=1 $l_{\text{air}} l^{-1} \text{min}^{-1}$ and 80% (v/v) oxygen, high lovastatin titers
25 were obtained, up to 300 mg l^{-1} . Upon this, they claimed that in the bubble column
26 it was insufficient to aerate the medium with air alone, as on average even five-
27 fold increase of lovastatin concentration could be achieved when air enriched with
28 pure oxygen was used [41]. Unfortunately, these authors supplied no information
29 about the changes of dissolved oxygen concentration in the medium in the
30 dependence on the aeration gas composition used. The only data that could make
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6 the issue of oxygen utilization clearer were the changes of specific oxygen uptake
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8 rate (SOUR). The SOUR decreased with time showing approximately the same
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10 profile from about 1 mmol O₂ g⁻¹ h⁻¹ in the early hours of the process down to
11
12 about 0.1 mmol O₂ g⁻¹ h⁻¹ at about 200 hour, irrespective of the fact, whether there
13
14 was 80% (v/v) of oxygen in aeration gas or not. Thus, it was unclear what
15
16 mechanism was responsible for the better lovastatin formation under oxygen
17
18 enriched conditions. Rodriguez Porcel et al. claimed that oxygen was required in
19
20 the post-PKS stage of lovastatin biosynthesis [41]. Unfortunately, these authors
21
22 did not refer to the formation of octaketide metabolites, as sulochrin or (+)-
23
24 geodin, which require molecular oxygen in two or three post-PKS steps (Fig. 2).
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30 Lai et al. tested a mutant which originated from ATCC20542 strain and
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32 showed in their experiments that the oxygen saturation profile was quite typical in
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34 the cultivation of *A. terreus*. There was a fast decrease within the first 24 hours,
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36 which reflected the exponential growth of biomass. When the dissolved oxygen
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38 level reached 30%, a cascade control using agitation was used to maintain the
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40 saturation level. If uncontrolled, the dissolved oxygen might have decreased down
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42 to zero before the idiophase [42].
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46 Hajjaj et al. compared volumetric oxygen uptake rates (OUR) in the
47
48 aforementioned complex and synthetic media. It occurred that in the complex
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50 media OUR exceeded 75 mmol O₂ l⁻¹ h⁻¹ in the end of trophophase, while in the
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52 synthetic media it was not higher than 18 mmol O₂ l⁻¹ h⁻¹. Lovastatin formation
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54 was higher in complex media, however there was no proof that it was due to
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56 higher oxygen uptake and not to nutrient composition [28]. Novak et al. claimed
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6 that the optimum oxygen saturation in the batch culture with glucose as a carbon
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8 source was 70%. Either too high (80%) or too low (35%) did not assure good
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10 lovastatin titer [37].

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12 Bizukojc and Ledakowicz tested lovastatin biosynthesis in batch and fed-
13
14 batch processes using three constant aeration rates [36]. Within contrast to Casas
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16 Lopez et al. [34], it was found that higher aeration rate enhanced (+)-geodin,
17
18 instead of lovastatin formation in batch cultures (Fig. 7). Higher aeration rate
19
20 increased organic nitrogen uptake rate and the decrease of pH in the early
21
22 idiophase was faster. Final (+)-geodin concentration was ten times higher, when
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24 vvm was increased from 0.308 to 0.513 $\text{l}_{\text{air}} \text{l}^{-1} \text{min}^{-1}$. Lovastatin titer was then
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26 four-fold lower. When aeration rate was increased further (up to 1.026 $\text{l}_{\text{air}} \text{l}^{-1} \text{min}^{-1}$
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28 ¹) in the lactose-fed fed-batch culture, the final (+)-geodin titer exceeded 200 mg l^{-1}
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30 ¹, while lovastatin concentration decreased to less than 10 mg l^{-1} [36].

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36 This effect of aeration was also seen when one compared the rotary shake
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38 flask culture run with bioreactor runs. Bizukojc and Ledakowicz found that at
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40 $\text{YE}_0 = 2 \text{ g l}^{-1}$ and $\text{LAC}_0 = 20 \text{ g l}^{-1}$ yield coefficient $Y_{\text{GEOD/LAC}}$ was equal to 3.76 mg
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42 GEOD g LAC^{-1} in shake flask culture and 12.3 mg GEOD g LAC^{-1} in the
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44 bioreactor at $\text{vvm} = 0.308 \text{ l}_{\text{air}} \text{l}^{-1} \text{min}^{-1}$. At the same time yield coefficient $Y_{\text{LOV/LAC}}$
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46 was equal to 4.34 mg LOV mg LAC^{-1} and 4.83 mg LOV mg LAC^{-1} in the
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48 corresponding shake flask and bioreactor runs [35,36]. It is clear that the aeration
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50 in the shake flask culture is poor compared to that of a stirred tank bioreactor.
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55 The elevated (+)-geodin titers induced by the increase of aeration rate were
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57 probably due to the fact that in the post-PKS tailoring steps leading to (+)-geodin
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6 formation there is a higher demand for molecular oxygen. Emodin anthrone
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8 oxygenase, questin oxygenase, dihydrogeodin oxidase are the enzymes requiring
9
10 the molecular oxygen as a cofactor in the (+)-geodin biosynthetic pathway. In the
11
12 lovastatin post-PKS pathway there is one hydroxylation and one oxidation step.
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14 Only the latter requires molecular oxygen [36].
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18 A completely different approach to investigate the effect of oxygen on
19
20 lovastatin biosynthesis was applied by Lai et al. [43]. They tested the influence of
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22 the organic oxygen carriers on the process. Such oxygen carriers are usually liquid
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24 n-alkanes of 12 to 16 carbon atoms in the chain.
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28 A 2.5% addition of dodecane to the medium in shake flask culture
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30 increased lovastatin titer from 100 up to 400 mg l⁻¹. The adverse effect was
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32 observed in a bioreactor, where the 4-fold decrease of lovastatin concentration
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34 was found [43]. Lai et al. found that the addition of dodecane increased dissolved
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36 oxygen of up to 70%, while in the dodecane-free run it was on the level of 20%.
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38 Probably, in this case the effect was similar to the one reported by Bizukoje and
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40 Ledakowicz [36]. The higher oxygen saturation in the medium did not facilitate
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42 lovastatin formation in the bioreactor. Nevertheless, Lai et al. [43] reported
43
44 nothing concerning the by-products of either sulochrin or (+)-geodin, so the effect
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46 of organic oxygen carriers on these metabolites cannot be discussed.
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51 The most important information on the influence of oxygen on the
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53 formation of secondary metabolites in *A. terreus* are summarized in Table 3.
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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 then 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

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6 dissolved oxygen levels [44]. No direct correlation was found but all profiles
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8 looked similar; initial pH increased up to 7.6, thereafter a decrease down to the
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10 different constant values between 6.5 and 7.2 was observed [44].
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13 The experiments with pH control using hydrochloric acid and sodium
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15 hydroxide in the production phase (about 48 hour) were not successful [44].
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17 Setting pH at three different levels 5.5, 6.5 and 7.5 did not supply any satisfactory
18
19 results as lovastatin titer, biomass content and biomass growth rate were lower in
20
21 all these runs in comparison to the pH-uncontrolled run.
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25 The more promising results concerning pH control in lovastatin production
26
27 were obtained by Bizukojc and Ledakowicz [36]. They used the same nutrients as
28
29 Lai et al. [44] but at lower nutrient concentrations (lactose 20 g l⁻¹ and yeast
30
31 extract 2 and 4 g l⁻¹). Also, pH control was started as soon as 24 hours and set at
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33 the levels of 7.6 and 7.8, depending on the initial yeast extract concentration.
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35 Owing to this procedure pH did not drop in the idiophase (Fig. 8). As a result of
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37 pH control lovastatin formation was practically unchanged and (+)-geodin
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39 formation was strongly aggravated. The important fact was that only the solution
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41 of sodium and potassium carbonates was used for pH control. The use of
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43 carbonates can be profitable as several reactions of the primary metabolism of *A.*
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45 *terreus*, including the one, which forms malonyl-CoA, are hydrocarbonate
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47 dependent [36].
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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:

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

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5 and it is valid in the range of n_{spores} from $1.39 \cdot 10^9 \pm 2.89 \cdot 10^8 \text{ l}^{-1}$ to
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8 $2.56 \cdot 10^{10} \pm 9.64 \cdot 10^9 \text{ l}^{-1}$ [47].
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10 The basic parameter to describe the morphology of the pellet-growing
11 fungi is pellet diameter. Because fungal pellets can be either smooth or hairy,
12 another morphological measure is also applied to evaluate pellet morphology. It is
13 the filament ratio, which is defined as the ratio between the areas of the peripheral
14 hairy surface of the pellet and the total area of the pellet [48]. Filament ratio
15 represents the extent to which free filaments grow out of the dense structure of the
16 pellet. This parameter was widely used by the researchers from University of
17 Almeria (Spain), who dealt with the changes of *A. terreus* morphology in
18 lovastatin biosynthesis [34,41,48,49,50]. Their research was, however, directed
19 more into the correlation of morphology with the aeration of the medium and its
20 rheological properties than with lovastatin yield. In our opinion the influence of
21 the morphology on the rheological properties of the media is beyond the scope of
22 this review and will not be discussed further. Only the hyphal morphology alone
23 and its correlation with lovastatin formation, if found, will be discussed.
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43 Rodriguez Porcel et al. [48] tested the morphology of *A. terreus* in a
44 fluidized bed reactor (FBR) as well as a stirred tank bioreactor (STB). In FBR
45 pellets had higher diameters of up to 2.5 mm, while in STB only 0.5-1 mm pellets
46 were observed. This was obviously attributed to the increased shear forces in the
47 STB. However, if the culture was aerated with oxygen-enriched (80% v/v) gas
48 phase even in the STB, pellet diameter exceeded 2 mm. The filament ratio
49 decreased from 1 down to 0.4, irrespective of the bioreactor, excluding the runs in
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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

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6 lovastatin yield decreasing it down to 28% of the value obtained in the control
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8 runs (non-sonicated) [50].
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10 Casas Lopez et al. used a relatively old 48 hour inoculum with large
11 diameter pellets (1.2 mm) [34]. They aimed to determine the effect of various
12 hydrodynamic conditions on pellet size in the stirred tank bioreactor. Pellets
13 increased in size (between 2 and 2.5 mm) under low agitation (300 rpm). At
14 higher agitation the pellets initially grew to 1.5 mm. Thereafter pellet diameter
15 was observed to reduce in size (0.9 mm) and was attributed to shear stress. No
16 direct quantitative correlation between mycelial morphology and lovastatin
17 production was shown in these experiments. Casas Lopez et al., however, claimed
18 that the loose pellets of more filamentous morphology assured better lovastatin
19 titers in comparison to the denser smaller pellets [34].
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34 In contrast Gupta et al. claimed that the middle sized pellets of diameter,
35 from 1.8 to 2 mm, were optimal for lovastatin biosynthesis [51]. They also
36 claimed that growing external cells were responsible for lovastatin biosynthesis,
37 although they showed no direct evidence of this. Despite this, this correlation was
38 confirmed by Bizukoje and Ledakowicz, and will be presented further [47].
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46 The number of spores introduced to the preculture is one of the most
47 sensitive parameters influencing the morphology of hyphae and biosynthesis of
48 secondary metabolites [52]. Tucker and Thomas observed that the increase of the
49 number of *P. chrysogenum* spores above $5 \cdot 10^8$ per liter caused the transition from
50 pelleted to dispersed growth [52]. Hyphal morphology and xylanase biosynthesis
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6 by *A. awamori* in relation to the initial number of spores was also investigated by
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8 Smith and Wood [53].
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10 In *A. terreus* similar relations were observed by Bizukojc and Ledakowicz
11 [47]. The general rule was confirmed that the more spores that were introduced
12 resulted in more pellets being formed at a reduced pellet diameter [47,52]. When
13 the initial number of *A. terreus* spores in the preculture was equal to $2.56 \cdot 10^{10} \text{ l}^{-1}$
14 the obtained pellets had on average diameter three times lower than for the case
15 when only $1.39 \cdot 10^9$ spores per liter were introduced (Fig. 9a). The size of pellets
16 influenced lovastatin formation. The smaller pellets were more efficient for
17 lovastatin biosynthesis (Fig. 9a). A correlation was not clear for (+)-geodin
18 formation until after 120 hours of cultivation and seemed to be independent of the
19 initial number of spores and subsequently pellet diameter. At this time it became
20 clear that (+)-geodin production was enhanced under conditions with a low spore
21 concentration [47].
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39 In the research concerning the morphology of fungal mycelium one can go
40 further than to measure size and shape of the filaments or pellets. The
41 differentiation of hyphae into apical, subapical and hyphal cells is another
42 important feature of the fungal growth, which has an impact on metabolite
43 formation. Whereas there are a lot of experimental data about the differentiation
44 of mycelium in the fungi which usually grow freely dispersed, such as *P.*
45 *chrysogenum*, or other filamentous organisms (*Streptomyces* sp.), the research
46 concerning pellet-forming fungi was less frequently performed, as it is more
47 complicated from a methodological point of view [45].
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6 There are few papers which focus on the differentiation of the pellets
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8 formed by filamentous organisms [54-56]. The work of Hamanaka et al. mainly
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10 focused on the visualization techniques of intracellular lipids in the pellets of
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12 *Mortierella alpina* [55] and Park et al. described the visualization technique for
13
14 the quantification of the intrastucture of tylosin-producing *Streptomyces fradiae*
15
16 pellets [54]. Freudenberg et al. supplied some details about the differentiation of
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18 hyphae in *A. awamori* pellets. They distinguished two zones in the pellets,
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20 growing and non-growing, with the use of a fluorescent stain, acridine orange
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22 [56].
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27 For a lovastatin producer namely *A. terreus* Bizukojc and Ledakowicz
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29 investigated the differentiation of pellets [47]. The pellets were stained with
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31 methyl blue and, having been processed, sectioned into thin slides. Two zones
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33 were distinguished and quantitatively evaluated as zone fractions. They comprise
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35 of growing Z_1 (apical) and non-growing Z_2 (hyphal) cells (Fig 9b and 9d). The
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37 growth of the active zone already started, when germ tubes emerged from spore
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39 agglomerates in the preculture (Fig. 9b). In that time growing cells fraction Z_1
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41 increased and the kernel of the pellet (hyphal cells fraction Z_2) was rather small.
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43 Since the transfer of the evolved pellets into the cultivation medium, the fraction
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45 of the active zone Z_1 decreased and the hyphal internal part of the pellets Z_2
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47 (kernel) dominated (Fig. 9b). Thereby, the metamorphosis of active cells into the
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49 hyphal cells took place. Also these were the hyphal cells, which finally
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51 contributed to the increase of biomass concentration expressed as dry weight,
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53 which was proved by the linear correlation shown in Fig. 9b [47].
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6 Bizukojc and Ledakowicz claimed that specific lovastatin and (+)-geodin
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8 formation rates correlated with active zone fraction. They found that specific
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10 lovastatin formation rate decreased linearly with the decrease of active, apical
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12 zone fraction Z_1 , while specific (+)-geodin formation rate increased exponentially
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14 (Fig. 9c).
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20 **Biomass growth and product formation kinetics in *Aspergillus terreus***

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22 The kinetic modeling of lovastatin biosynthesis was seldom the object of
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24 research. Many authors showed only the graphs with the changes of lovastatin
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26 concentration in time. However, upon analyzing these data, one may conclude that
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28 lovastatin biosynthesis, unlike many other secondary metabolites, is varied in
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30 terms of growth and non-growth association i.e. mixed-growth associated. The
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32 inspection of the graphs in [27,30,33,41,44] clearly showed that lovastatin
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34 production started already in the biomass growth phase (trophophase) and was
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36 prolonged into the idiophase, especially if there was enough carbon source left in
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38 the medium or a feeding strategy was applied. Only Hajjaj et al. showed the
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40 graph, in which lovastatin appeared in the medium in the late idiophase and thus
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42 an impression may have occurred that its formation was non-growth associated
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44 [28].
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51 The detailed analysis of product growth association was made upon the
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53 time evolution of product and biomass volumetric formation rates, as well as upon
54
55 the relation between specific biomass and product formation rates [31, 35]. At a
56
57 glance, lovastatin formation was mainly associated with biomass growth.
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6 Although, the cease of biomass growth did not prevent lovastatin formation, if
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8 carbon source was available (Fig. 6). Bizukojc and Ledakowicz showed that the
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10 maxima of biomass and lovastatin volumetric formation rates did not ideally
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12 coincide (Fig 10a) and the delay in lovastatin formation in the trophophase was
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14 observed. The relation between specific lovastatin formation rate and specific
15
16 biomass growth rate was not linear either (Fig 10b). The reason of this
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18 phenomenon was that at the highest biomass growth rates lovastatin is not
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20 produced due to the high nitrogen levels (nitrogen inhibition). This lag in
21
22 lovastatin formation with the increase in initial nitrogen content is noted in Fig 3.
23
24 Thus, one sometimes could have an impression that lovastatin formation is
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26 delayed till as late as the idiophase, which in the extreme case may provoke the
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28 conclusion that lovastatin formation is practically non-growth associated. In fact,
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30 it is mixed-growth association and this way the real reason of this phenomenon
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32 connected with elevated nitrogen level in the beginning of the process may remain
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34 unnoticed [35].
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41 In case of (+)-geodin, the situation is completely different. Bizukojc and
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43 Ledakowicz observed that this metabolite was excreted extensively in the
44
45 idiophase and there was no association between its formation and biomass growth.
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47 The yield coefficient defined as the increment of product divided by the increment
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49 of biomass was equal to infinity for (+)-geodin [36].
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53 There are only two papers published, which focused on the kinetic
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55 modeling of *A. terreus* growth and lovastatin formation. Liu et al. proposed a
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57 morphologically structured model for biomass growth, hyphal differentiation and
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6 lovastatin formation by *A. terreus* [57]. This model was very similar to the
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8 morphologically structured model previously shown for penicillin biosynthesis
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10 [56]. Liu et al. assumed the inhibitive effect of glucose originated from the
11
12 enzymatic hydrolysis of starch, the carbon source used, but omitted the inhibitive
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14 effect of organic nitrogen, which is rather a mistake [57]. Additionally, they did
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16 not supply their own data on the hyphal differentiation but used the kinetic
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18 parameters from *P. chrysogenum* [58]. The assumption that these parameters are
19
20 close to that of *A. terreus* is inaccurate and could lead to erroneous results. There
21
22 is no evidence that the differentiation kinetics and morphological form of both
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24 fungi are similar. As mentioned in the previous section, *A. terreus* is known to
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26 evolve in the form of macroscopic pellets, while *P. chrysogenum* favors dispersed
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28 hyphae. Liu et al. failed to mention the morphological form of the fungus in their
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30 experimental runs [57].
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36 Bizukojc and Ledakowicz proposed a simple unstructured kinetic model
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38 for lovastatin biosynthesis upon the following assumptions evolved from the
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40 detailed research on the influence of the initial lactose and yeast extract
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42 concentrations on the process. Thus lactose was the sole carbon source and yeast
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44 extract the sole organic nitrogen source. The excess of organic nitrogen exerted an
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46 inhibitive effect on lovastatin biosynthesis and lactose uptake. Lovastatin
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48 biosynthesis was mixed-growth associated, so lovastatin balance consisted of two
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50 terms: biomass growth associated and biomass non-growth associated lovastatin
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52 formation. Lactose was both utilized for biomass formation and lovastatin
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54 formation. Lactose was both utilized for biomass formation and lovastatin
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56 biosynthesis [31].
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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

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

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13 • The influence of pH on lovastatin biosynthesis is generally not of the
14
15 highest importance. One agrees that initial pH should be about 6.5. The
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17 profile of pH depends on the medium composition and the control of pH
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19 rather does not lead to the increase in lovastatin production.
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22 • The formation of **other polyketide** metabolites by *A. terreus* in **the** lovastatin
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24 process has been underestimated in the research so far. The formation of the
25
26 most profound by-product (+)-geodin is strongly carbon-dependent, thus
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28 facilitated in C-source-fed fed-batch **cultivations**, and **is** more sensitive to
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30 elevated organic nitrogen levels. Furthermore, **high aeration rates of the**
31
32 **broth increase** (+)-geodin formation. The control of pH at the level slightly
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34 above neutral leads to the decrease of (+)-geodin production.
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36
37 • Few correlations between the formation of polyketide metabolites by *A.*
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39 *terreus* and morphology of hyphae can be found. The smaller pellets
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41 (diameter lower than 2 mm) are the most favorable for lovastatin
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43 biosynthesis. No correlation between pellet size and (+)-geodin formation is
44
45 found. Lovastatin formation is also connected with the metamorphosis of
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47 hyphae and its specific formation rate is linearly dependent on the amount
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49 of growing cells. With regard to (+)-geodin, its formation is not correlated
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51 with the presence of active cells.
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6 • From the kinetic point of view lovastatin formation is mixed-growth
7 associated. Its biosynthesis is extensive already in the trophophase but can
8 be continued in the idiophase, if sufficient amount of carbon is present in
9 the medium. Lovastatin biosynthesis may be delayed, if too much organic
10 nitrogen is present in the medium. Nitrogen starvation is believed to be
11 favorable for both lovastatin and (+)-geodin biosynthesis. As opposed to
12 lovastatin, (+)-geodin formation is non-growth associated. This metabolite
13 is excreted to the medium in the idiophase and this process is especially
14 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-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 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

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6 concentrations 20 and 4 g l⁻¹, lactose concentration in the feed equaled 100 g l⁻¹
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8 [35]

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

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

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

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27 Fig. 10. Association of lovastatin and (+)-geodin with biomass growth at *A.*
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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	YE or SM or CSL (0.144-0.172 g N l ⁻¹)	40-120 mg LOV l ⁻¹	ATCC20542	[30]
GLY (20.44 g l ⁻¹)				
LAC (20 g l ⁻¹)				
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	ATCC20542	[31]
LAC (5-40 g l ⁻¹)	YE (2-12 g l ⁻¹)	5-110 mg LOV 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,

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])

Supplemen- tation	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	ATCC20542	[41]	
	1.0	20%**				25 mg LOV l ⁻¹
		80%**				10-50 mg LOV l ⁻¹
	1.5	80%**				50-300 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

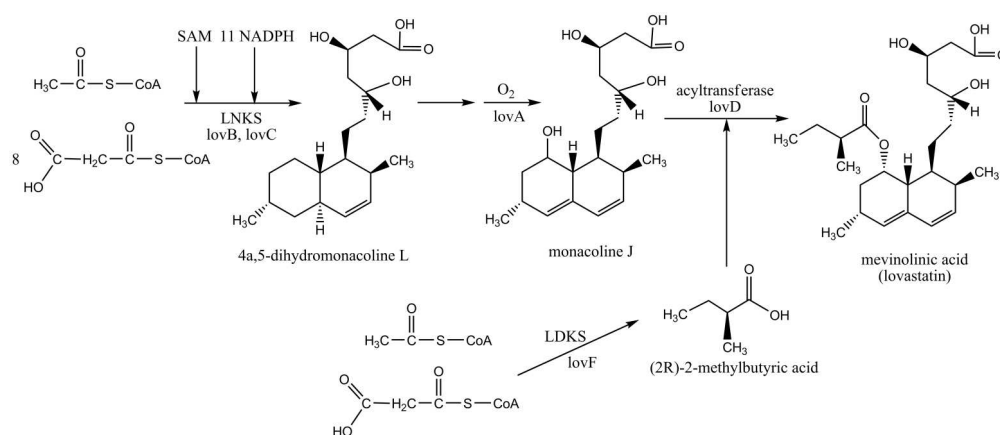


Fig.1 Simplified scheme of lovastatin biosynthesis. Abbreviations used: SAM ♦ S-Adenosyl-L-Methionine, LNKS ♦ Lovastatin NonaKetide Synthase, LDKS - Lovastatin DiKetide Synthase, lovA, lovB, lovC, lovD, lovF ♦ genes responsible for coding of the presented enzymes. For the detailed information concerning 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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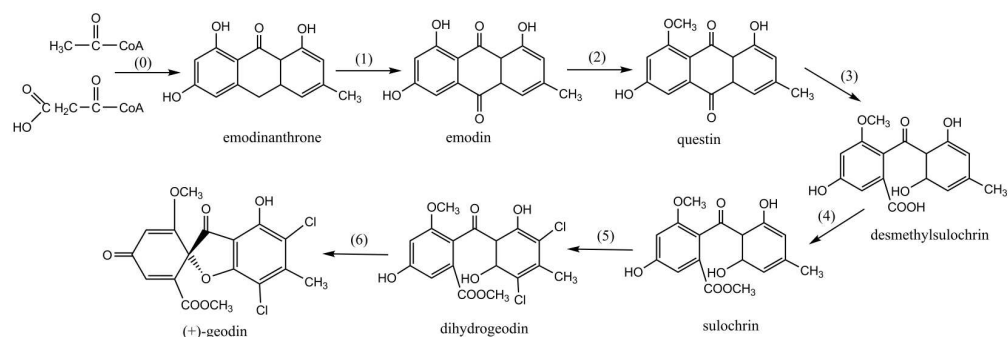


Fig.2. Simplified scheme of (+)-geodin biosynthesis upon [14]. The enzymes catalyzing the reactions: (0) uncharacterized octaketide synthase, (1) emodin anthrone oxygenase, (2) emodin O-methyltransferase (3), questin oxygenase, (4) desmethylsulochrin O-methyltransferase, (5) chloroperoxidase, (6) dihydrogeodin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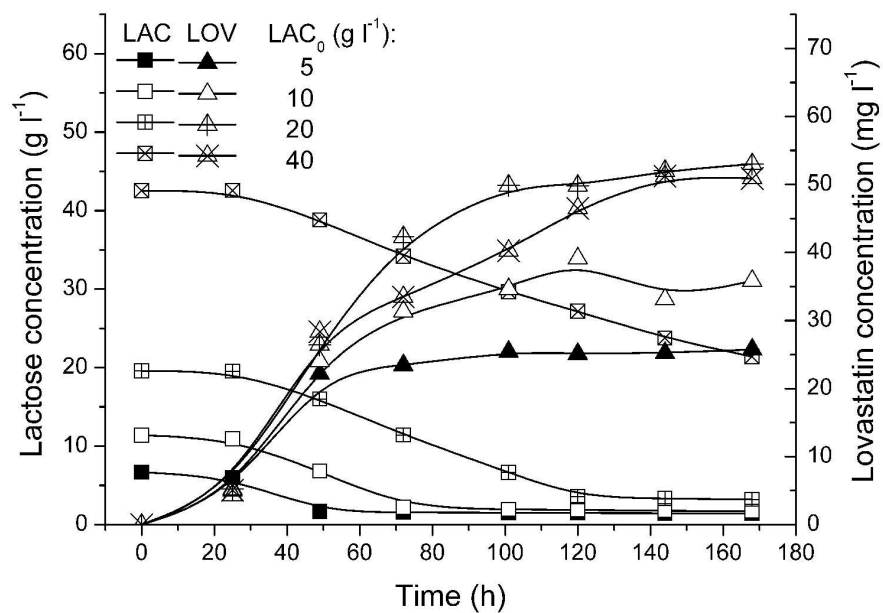


Fig.3. Lovastatin (LOV) biosynthesis at the varying initial lactose (LAC₀) concentration; initial yeast extract concentration set at 4 g l⁻¹ [31]
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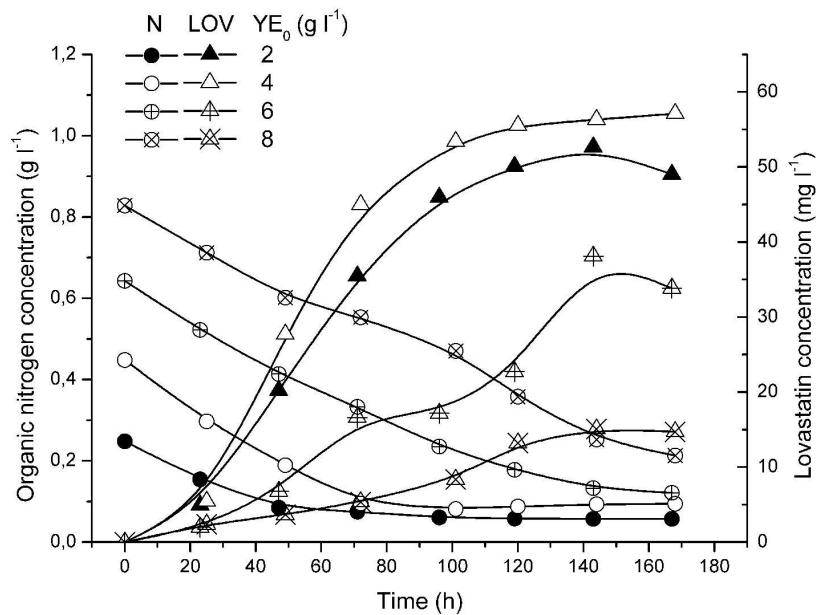


Fig.4 Lovastatin (LOV) biosynthesis and organic nitrogen (N) utilization at the varying initial yeast extract (YE₀) concentration; initial lactose concentration set at 20 g l⁻¹ [31]
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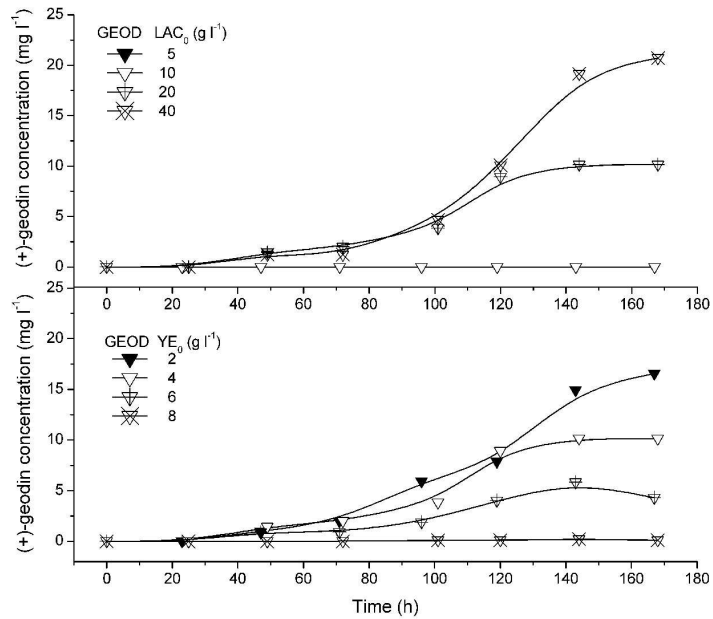


Fig.5 (+)-geodin (GEOD) biosynthesis at the varying initial lactose (LAC₀) and yeast extract (YE₀) concentrations; initial yeast extract and lactose concentrations equal to 4 and 20 g l⁻¹ respectively

[35]

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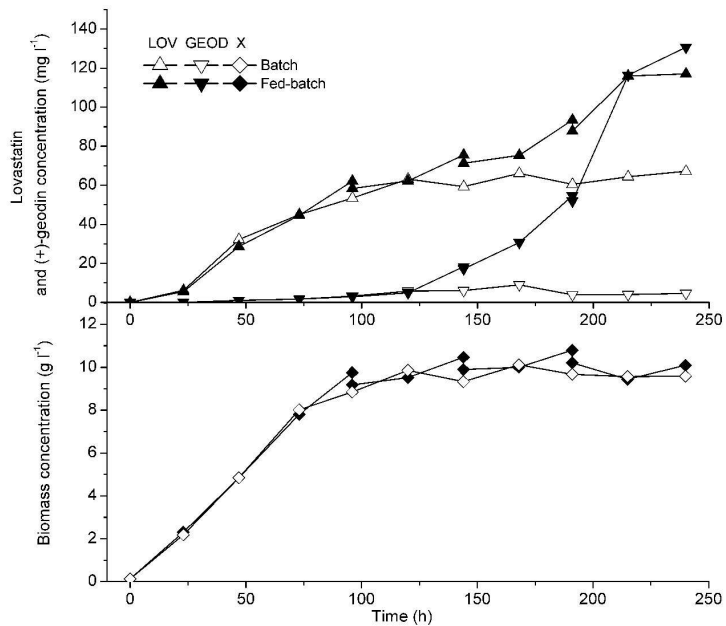


Fig.6 Lovastatin and (+)-geodin biosynthesis in batch and lactose-fed discontinuous fed-batch shake flask culture; initial lactose and yeast extract concentrations 20 and 4 g l⁻¹, lactose concentration in the feed equaled 100 g l⁻¹ [35]
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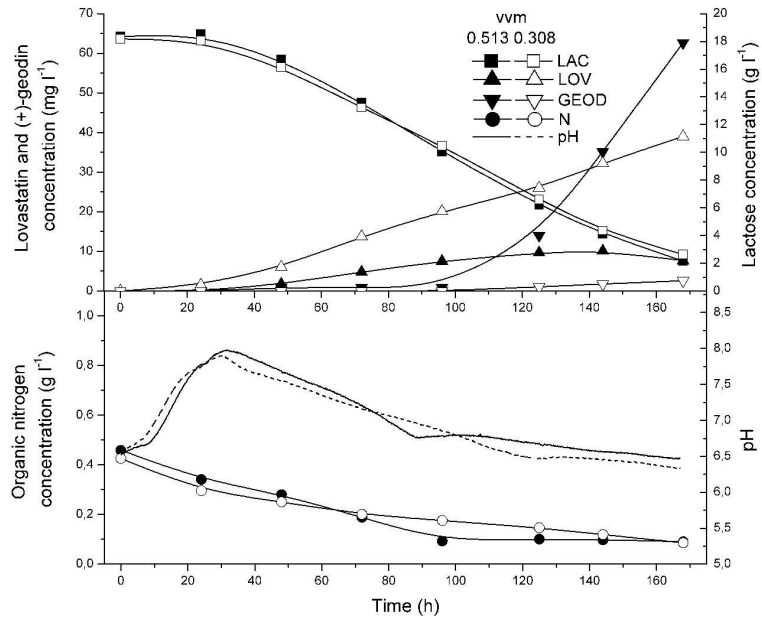


Fig.7 Influence of the aeration rate on lovastatin and (+)-geodin biosynthesis in a stirred tank bioreactor; initial lactose and yeast extract concentration 20 g l⁻¹ and 4 g l⁻¹ [36]
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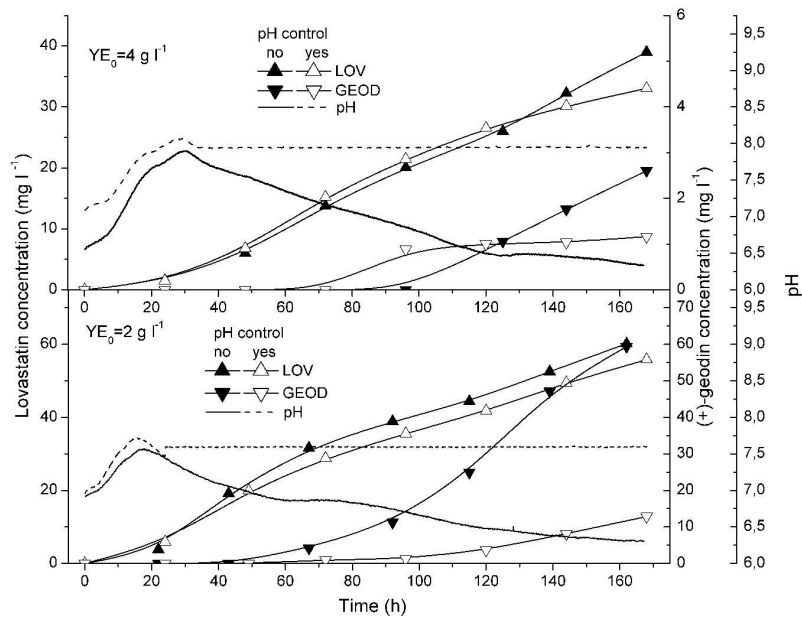


Fig.8 Influence of pH control and initial yeast extract concentration on lovastatin biosynthesis in the bioreactor; initial lactose concentration 20 g l^{-1} [36]
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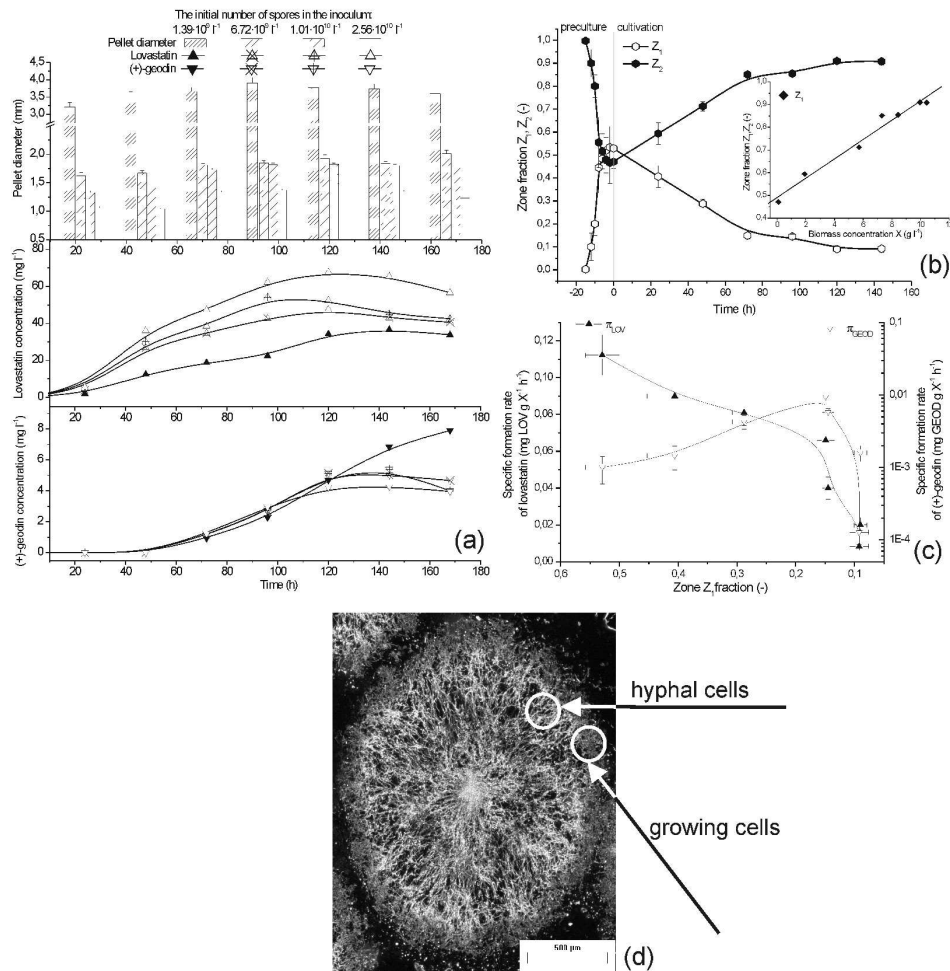


Fig. 9 Influence of the initial number of spores in the preculture on pellet morphology and product formation (a) and differentiation of *A. terreus* hyphae (b); hyphal differentiation vs. product formation rate (c) in lovastatin and (+)-geodin biosynthesis; the cross section of *A. terreus* pellet (d): 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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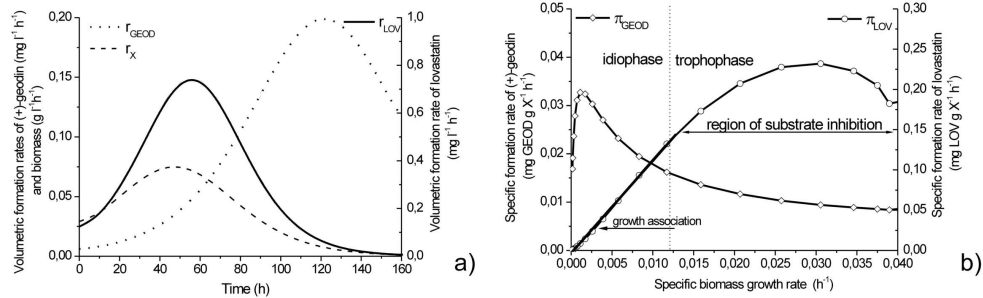


Fig.10 Association of lovastatin and (+)-geodin with biomass growth at *A. terreus* determined upon the time changes of biomass (r_X), lovastatin (r_{LOV}) and (+)-geodin (r_{GEOD}) volumetric formation rates (a) and the relationship between specific lovastatin (π_{LOV}) and (+)-geodin (π_{GEOD}) formation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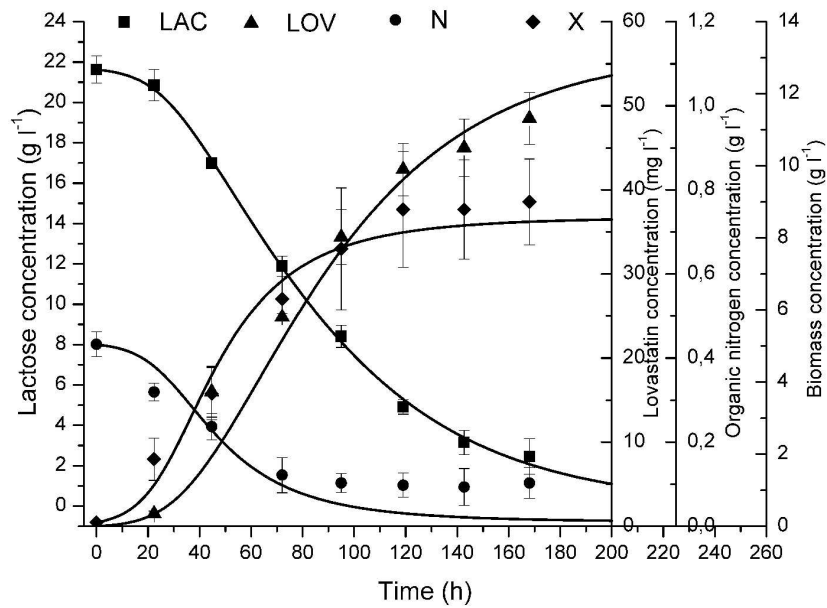


Fig.11 One of the fits of the model (eqs. from 2 to 5) to the experimental data; initial lactose concentration 20 g l⁻¹, initial yeast extract concentration 4 g l⁻¹ [31]
286x201mm (600 x 600 DPI)