Impact of bioreactor scale on lovastatin biosynthesis by *Aspergillus terreus* ATCC 20542 in a batch culture

Marta Pawlak[*], Marcin Bizukojć, Stanisław Ledakowicz

Technical University of Lodz, Faculty of Process and Environmental Engineering, Department of Bioprocess Engineering, ul. Wólczańska 213, 90-924 Łódź, Poland

Biosynthesis of lovastatin (a polyketide metabolite of *Aspergillus terreus*) in bioreactors of different working volume was studied to indicate how the change of scale of the process influences the formation of this metabolite. The experiments conducted in shake flasks of 150 ml working volume allowed to obtain lovastatin titres at the level of 87.5 mg LOV l^{-1}, when two carbon sources, namely lactose and glycerol were used. The application of the same components in a large stirred-tank bioreactor of 5.3-litre working volume caused a decrease of lovastatin production by 87% compared to the shake flask culture. The deficiency of nitrogen in this bioreactor did not favour the formation of lovastatin, in contrast to the small bioreactor of 1.95-litre working volume, in which lovastatin titres comparable to those in the shake flasks could be achieved, when organic nitrogen concentration was two-fold decreased. When the control of *pH* and/or *pO₂* was used simultaneously, an increase in lovastatin production was observed in the bioreactors. However, these results were still slightly lower than lovastatin titres obtained in the shake flasks.

Keywords: lovastatin, *Aspergillus terreus* ATCC 20542, shake flask, stirred tank bioreactor, batch process

1. INTRODUCTION

Lovastatin ($C_{24}H_{36}O_5$) is a secondary polyketide metabolite produced by such filamentous fungi as *Aspergillus terreus* and *Monascus ruber*. This compound is a natural statin, commonly used as a sovereign antihypercholesterolemia drug. The effect of lovastatin is revealed through the inhibition of (S)-3-hydroxymethylglutaryl-CoA (3-HMG-CoA) reductase, which converts 3-HMG-CoA to mevalonate in the pathway of cholesterol biosynthesis in human cells. The basic strain of *A. terreus* capable of the formation of this metabolite was denoted as ATCC 20542 by American Type Culture Collection.

In many studies this primary producer of lovastatin, i.e. *A. terreus* ATCC 20542, was used. There are several reports concerning it, both in shake flasks and bioreactors, in which different media and process conditions were used. In the experiments performed in shake flasks a significant impact on the course of lovastatin biosynthesis had, first of all, type and concentration of carbon and nitrogen sources (Bizukojć and Ledakowicz, 2007a; Casas Lopez et al., 2003). In bioreactors it was also necessary to use optimal *pH* (Bizukojć and Ledakowicz, 2008; Lai et al., 2005), oxygen saturation of broth and aeration rate (Casas Lopez et al., 2005; Lai et al., 2005; Rodriguez Porcel et al., 2006). Lovastatin titres obtained with ATCC 20542 were usually moderate, compared to those, which were the effect of the application of other strains, including the mutant ones (Hajjaj et al., 2001; Kumar et al., 2000; Manzoni et al, 1998).

---

*Corresponding author, e-mail: marta.pecyna85@o2.pl

Lai et al. (2002) carried out experiments in shake flasks and in a 5-litre bioreactor using ATCC 20542 strain. They studied the effect of oxygen carrier addition (aliphatic hydrocarbons) at various concentrations (0-5%, *w/v*) to the medium. They claimed that oxygen carrier enhanced lovastatin biosynthesis only in shake flasks, while in the bioreactor the contrary effect occurred. These findings were some of the important prerequisites on different behaviour of *A. terreus* in the shake flask culture and bioreactor. The same authors also tested the effect of *pH* and dissolved oxygen control in lovastatin biosynthesis by *A. terreus* ATCC 20542 (Lai et al., 2005). The investigations conducted in a 3-litre batch bioreactor showed that the optimal level of dissolved oxygen (*DO*) was equal to 20% and *pH* control decreased lovastatin concentration in the broth.

Different results were obtained by Casas Lopez et al. (2004). They proved that by increasing oxygen content in the aeration gas from 20% to 80% (*v/v*) a four times higher lovastatin concentration of about 80 mg LOV l^{-1} can be obtained in a shake flask culture. The same researchers later confirmed these results conducting experiments with oxygen-enriched air (80% *v/v* oxygen) in a 20-litre bubble column bioreactor.

The influence of carbon and nitrogen sources on the metabolism of *A. terreus* ATCC 20542 in shake flasks was examined by both Casas Lopez et al. (2003) and Bizukojć and Ledakowicz (2007a). They reported that nitrogen deficiency was favourable for lovastatin biosynthesis in a shake flask culture. However, too low nitrogen concentration may result in a decrease of biomass concentration and consequently a decrease of the titre of lovastatin whose formation is to a large extent growth associated (Bizukojć and Ledakowicz, 2007b). At the same time in the bioreactor lovastatin was better produced at extremely low nitrogen levels. (Bizukojć and Ledakowicz, 2008).

Summing up, it must be noticed that many studies on lovastatin production by *A. terreus* ATCC 20542 were made in shake flasks (Bizukojć and Ledakowicz, 2007a; Casas Lopez et al., 2003; Lai et al., 2002). Some of the presented results came from bioreactor cultures only (Bizukojć and Ledakowicz, 2008; Lai et al., 2005; Rodriguez Porcel et al., 2006). Thus, it is difficult to find a comparative study analysing simultaneously lovastatin biosynthesis both in shake flasks and bioreactors.

Therefore, this work aims at presenting how to increase the scale of lovastatin biosynthesis on the individual and two carbon sources: lactose and/or glycerol using bioreactors of different working volume starting from shake flasks of 150 ml up to two stirred tank bioreactors of 1.95-litre and 5.3-litre working volumes, in order to achieve comparable lovastatin titres in all these bioreactors.

2. MATERIALS AND METHODS

2.1. Strain and media

The strain *Aspergillus terreus* ATCC 20542 was employed in the experiments of lovastatin biosynthesis. They were conducted in the shake flasks of 150 ml working volume and in the stirred-tank bioreactors of 1.95-litre and 5.3-litre working volume, at 30°C. The speed of the rotary shaker was constant at 110 min^{-1}. In the bioreactor runs with pO_2 and *pH* control, the dissolved oxygen saturation was controlled at 20%, by changing the air flow rate and rotary speed of the impeller. The initial rotary speed of the impeller was from 150 to 200 min^{-1} dependent on the bioreactor. The control of *pH* was performed with either a solution of sodium and potassium carbonates or sodium hydroxide and kept at the levels between 7 and 7.8, dependent on the experiment. The preculture was prepared from spores grown on 10-days malt extract slants. The spores were washed, suspended in the preculture medium to achieve approximately 10^9 spores per litre and precultivated for 24 hours in the shake flasks. The inoculation was performed with the 24-hour preculture. The media contained the following mineral components: potassium dihydrophosphate KH_2PO_4: 1.51 g l^{-1}, magnesium sulphate $MgSO_4 \cdot 7H_2O$: 0.52

g l^{-1}, sodium chloride NaCl: 0.4 g l^{-1}, zinc sulphate ZnSO$_4$·7H$_2$O: 1 mg l^{-1}, ferric nitrate Fe(NO)$_3$·9H$_2$O: 2 mg l^{-1}, biotin: 0.04 mg l^{-1} and 1 ml solution of trace elements per 1 l of medium: The solution of trace elements contained sodium tetraborate Na$_2$B$_4$O$_7$·10 H$_2$O: 100 mg l^{-1}, manganese chloride MnCl$_2$: 50 mg l^{-1} sodium molybdate Na$_2$MoO$_4$·2H$_2$O: 50 mg l^{-1} and copper sulphate CuSO$_4$·5H$_2$O: 250 mg l^{-1} Yeast extract (BD, USA) was used as the nitrogen source at the concentration of 4 g l^{-1} and 2 g l^{-1} (8 g l^{-1} in the preculture) (Bizukojć and Ledakowicz, 2007a). Glycerol and/or lactose monohydrate were carbon substrates. All the process conditions are collected in Table 1.

Table 1. Experimental scheme for all runs

Run	$c_{LAC,0}$ [gl^{-1}]	$c_{GLY,0}$ [gl^{-1}]	$c_{YE,0}$ [gl^{-1}]	$c_{X,0}$ [gl^{-1}]	vvm [l$_{air}$ min^{-1} l^{-1}]	pH control	pO_2 control	Source of data
SF1	10	10		0.07	-	-	-	Bizukojć and Pecyna, 2011
SF2	0	20		0.06	-	-	-	Bizukojć and Pecyna, 2011
SF3	20	0	4	0.10	-	-	-	Bizukojć and Ledakowicz, 2007a
SB1	20	0		0.20	0.308	no	no	Bizukojć, Ledakowicz, 2008
SB2	20	0	2	0.24	0.308	no	no	Bizukojć and Ledakowicz, 2008
LB1	20	0		0.42	0.3	no	no	present study
LB2	10	10	4	0.21	0.283	no	no	present study
LB3	10	10	2	0.15	0.282	no	no	present study
LB4	10	10		0.29	0.283-0.998	at 7.15 with NaHCO$_3$, and KHCO$_3$	at 20%	present study
LB5	10	10	4	0.23	0.283-0.779	at 7.15 with NaOH	at 20%	present study
SF/A1	10	10		0.26	-	no	-	present study
SF/A2	10	10		0.26	-	with NaHCO$_3$ and KHCO$_3$*	-	present study

* pH level could not be set due to lack of pH measurement on-line. Instead, constant volumes of carbonate solution was added at equal intervals of 24 hours

2.2. Analytical methods

Lovastatin (*LOV*) and (+)-geodin (*GEOD)*, (a major by-product in lovastatin biosynthesis) were simultaneously analysed with HPLC (Waters, USA). The analytical conditions were as follows: a Waters Symmetry Shield RP18 column (4.6 mm×250 mm×5μm) at the gradient elution CH$_3$CN – 0.1% H$_3$PO$_4$ (40:60, *v/v*) up to 7 min and CH$_3$CN – 0.1% H$_3$PO$_4$ (60:40, *v/v*) from 8 to 30 min. The

temperature of the column was 25°C. Lovastatin was detected by a photodiode array detector at $\lambda=238$ nm and (+)-geodin, at $\lambda=280$ nm (Bizukojć and Ledakowicz, 2007b).

Lactose (*LAC*) and glycerol (*GLY*) were determined on an Aminex HPX-87H (BioRad, USA) column. The compounds were eluted with 0.01N H_2SO_4 at a flow rate of 0.6 ml min^{-1} and 60°C and detected by a refractive index detector. All the samples before HPLC analysis were filtered through 0.45 μm syringe filters with membranes made of regenerated cellulose.

Biomass (*X*) was assayed as dry weight. A 40 ml aliquot was filtered through paper filters and washed with a 10-fold amount of distilled water. Biomass was dried at 105°C to a constant weight. Organic nitrogen (*N*) was determined with the use of carbon and nitrogen analyser IL550TOC-TN (HACH, USA). Prior to the analysis the samples were 20-fold diluted to fit in the analytical range of the instrument.

The error of analytical methods used in the study did not exceed 3% for HPLC analyses and for organic nitrogen determination, and 8% for dry weight biomass assay.

3. RESULTS AND DISCUSSION

3.1. Lovastatin biosynthesis on the individual and two carbon sources in the shake flasks

It is generally believed in biochemical engineering that bioreactor cultivations are more efficient and more useful than shake flasks for several reasons. First of all, in many bioreactor systems, including stirred tank bioreactors, it is possible to control the factors, which may have significant influence on the course of the biosynthesis of a metabolite, e.g. *pH*, redox potential, aeration rate and/or oxygen saturation of the medium. Secondly, larger volumes of bioreactors compared with typical shake flasks allow to increase medium volume and thus higher biomass concentration can be applied. It is especially important, as in most of the cases the obtained metabolite concentration and its formation rate is biomass concentration dependent. Quite often it is also proportional to biomass growth rate (growth associated product formation or mixed associated product formation). Thirdly, broths in stirred tank bioreactors are better mixed improving the availability of all substrates. And last but not least, only bioreactors of various constructions can be practically applied in industrial processes.

As mentioned in the Introduction, in processes of lovastatin biosynthesis carried out in shake flasks, the type and concentration of carbon and nitrogen sources had a significant influence on its production. It is clearly evident in the results presented below and these results are going to be treated as reference values in further considerations concerning bioreactor processes.

In Fig. 1 a comparison of lovastatin concentration and lovastatin and (+)-geodin volumetric formation rates was presented for the runs with $c_{LAC,0}=20$ g l^{-1} (*SF3*) (Bizukojć and Ledakowicz, 2007a), $c_{GLY,0}=20$ g l^{-1} (*SF2*), and with a mixture of lactose and glycerol; $c_{LAC,0}=10$ g l^{-1} and $c_{GLY,0}=10$ g l^{-1} (*SF1*) (Bizukojć and Pecyna, 2011). At all these runs organic nitrogen concentration was the same as 4 g of yeast extract per litre was the sole nitrogen source. The selection of carbon and nitrogen substrate concentration was not incidental here, as it was previously shown that 20 g of lactose and 4 g of yeast extract per litre are optimum in the shake flask culture (Bizukojć and Ledakowicz, 2007a). It must be also mentioned here that glycerol has exactly the same amount of carbon as lactose monohydrate, so these are the equivalent substrates with regard to the amount of elemental carbon.

In the process with the initial glycerol concentration equal to 20 g GLY l^{-1}, only about 39 mg LOV l^{-1} was obtained. But when glycerol was replaced by lactose at the same concentration (20 g l^{-1}), higher lovastatin titre was found (an increase by 26%). The best results were, however, obtained using these

two carbon sources simultaneously, at about 144 hour of the run there was 87.5 mg LOV l^{-1} in the broth.
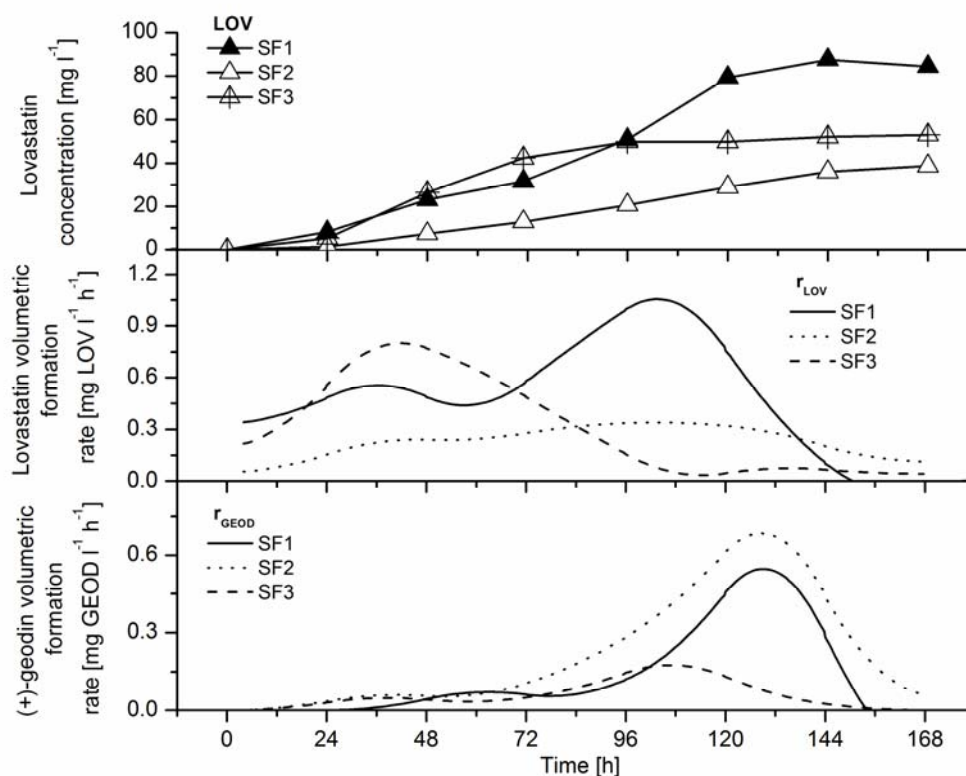

Fig. 1. Comparison of lovastatin concentration and lovastatin and (+)-geodin volumetric formation rates in the runs with $c_{LAC,0}$=20 g l^{-1}, $c_{GLY,0}$=20 g l^{-1}, and with the mixture of lactose and glycerol ($c_{LAC,0}$=10 g l^{-1} and $c_{GLY,0}$=10 g l^{-1}). Data for $c_{LAC,0}$=20 g l^{-1} come from Bizukojć and Ledakowicz (2007a). Data for $c_{GLY,0}$=20 g l^{-1} and for mixture of lactose and glycerol ($c_{LAC,0}$=10 g l^{-1} and $c_{GLY,0}$=10 g l^{-1}) come from Bizukojć and Pecyna (2011)

On the basis of lovastatin volumetric formation rate curves it can be noted that the utilisation of lactose in the medium in the first 48 hours of the run considerably favoured lovastatin biosynthesis. The maximum of lovastatin volumetric formation rate in the run with lactose was exactly at about 48 hour (0.80 mg LOV l^{-1} h^{-1}), while in the run with glycerol this maximum was significantly lower (0.34 mg LOV l^{-1} h^{-1}) and occurred not earlier than at 96 hour of the experiment. What is more, glycerol as the individual carbon source exerted an undesired effect of elevated (+)-geodin production. In the run with $c_{GLY,0}$=20 g l^{-1} (+)-geodin volumetric formation rate reached the highest values compared with the other two processes. In the run with two carbon sources lovastatin volumetric formation rate had two maxima and they were higher both for lactose and glycerol utilisation than in the runs in which these components were used as the individual carbon sources (Bizukojć and Pecyna, 2011).

Similar studies with the use of different carbon sources in shake flasks were performed by Casas Lopez et al. (2003). These authors obtained almost identical results as (Bizukojć and Pecyna, 2011) for lactose as carbon sources (1.33 g l^{-1} of yeast extract), about 90 mg LOV l^{-1}. What is surprising, the same high amount of lovastatin for glycerol was obtained (90 mg LOV l^{-1}), which is in disagreement with the results presented in Fig. 1.

The obtained levels of lovastatin in the shake flasks are satisfactory compared to the results of the other authors for *A. terreus* ATCC 20542 in similar conditions. Thus, at this moment, having obtained such good results as the ones in the shake flask culture, one faces the problem of how to achieve similar lovastatin titres in a bioreactor.

3.2. Lovastatin biosynthesis in stirred tank bioreactors of different working volumes

The experiments on lovastatin biosynthesis conducted in the shake flasks showed that a high lovastatin concentration (from 39 to 90 mg LOV l^{-1}) can be achieved using the optimum carbon (lactose and/or glycerol) and nitrogen (yeast extract) sources with their initial concentration equal to 20 g l^{-1} and 4 g l^{-1}, respectively. The difference in the quality of glycerol and lactose referred to lovastatin biosynthesis was discussed elsewhere (Bizukojć and Pecyna, 2011).

In order to test lovastatin production in the bioreactors and hopefully obtain higher lovastatin concentration a series of batch processes in stirred-tank bioreactors of 1.95- and 5.3-litre working volumes were performed. Initially, the following conditions: *vvm* equal to about 0.3 l_{air} min^{-1} l^{-1} no dissolved oxygen and *pH* control were set to somewhat follow the conditions in the shake flasks. In Fig. 2 lovastatin and lactose concentration, and lovastatin formation rates in the runs with $c_{LAC,0}$=20 g l^{-1} conducted in the shake flasks (reference run *SF3*), a small 1.95-litre bioreactor (*SB1*) and a large 5.3-litre bioreactor (*LB1*) working volume are presented. Despite the application of lactose at the same concentration in all the three processes, the highest lovastatin concentration (53 mg LOV l^{-1}) was obtained in the shake flasks (*SF3*), while the lowest (4.2 mg LOV l^{-1}) for the run in the 5.3-litre bioreactor (*LB1*). Also in the 1.95-litre bioreactor (*SB1*) the results were not as satisfactory (39 mg LOV l^{-1}) as they were in the shake flasks. It is not easy to explain this phenomenon. Its reason might probably be the level of oxygen dissolved in the medium. No pO_2 measurement could be made in the shake flasks due to the technical reasons, so actually it is difficult to compare the conditions in the flasks and bioreactor. Neither can the aeration rate be measured and controlled in the shake flasks. Convective mass transfer coefficient might not be so low in the shake flasks as expected, so the cells might have more oxygen available and thus bioreactor conditions were not properly set. On the other hand, it is clearly seen that volumetric lactose uptake rate for all these runs was approximately constant between 24 and 120 hour of each run and equal to 0.17, 0.12 and 0.15 g LAC l^{-1} h^{-1} for *SF3*, *SB1* and *LB1*, respectively. Only lower consumption of lactose between 24 and 72 hour in *SB1* in comparison to the other runs was observed. As volumetric substrate utilisation rate is always proportional to the amount of oxygen available and utilised (oxygen uptake rate), the hypothesis of better or worse aeration in these two runs is not so convincing. What is more, similar conclusions can be drawn from the analysis of nitrogen concentration curves. They were similar too and also in *SB1* slower consumption of nitrogen between the same hours was noted (Fig. 2). Also, the changes of substrate and biomass concentrations show no extreme differences between each other. The largest discrepancies are only seen for lovastatin curves. All in all, lovastatin formation is somewhat deteriorated in the bioreactor.

In order to finally confirm this negative effect in the bioreactor process, lovastatin volumetric formation rates were calculated. The highest value of the maximum of lovastatin volumetric rate (0.80 mg LOV l^{-1} h^{-1}) was observed in the run *SF3* (as described earlier in section *3.1. Lovastatin biosynthesis on the individual and two carbon sources in the shake flasks*). In run *SB1* this maximum occurred about 65 hour and was equal to 0.3 mg LOV l^{-1} h^{-1}, while in run *LB1* it was only 0.18 LOV l^{-1} h^{-1} (at about 32 hour).

It was shown previously (Bizukojć and Pecyna, 2011) that in shake flasks the use of two carbon sources led to more efficient lovastatin production, so another bioreactor process was conducted with the use of two carbon sources. The results are presented in Fig. 3 and they were unsatisfactory for the bioreactor run (*LB2*) again. Firstly, it is well seen that this time in the bioreactor slower consumption of carbon substrates occurred. Conversely, in the run *SF1* glycerol was fully utilised within 72 hours, then lactose was almost completely depleted within the next 48 hours. In the bioreactor glycerol consumption was delayed by at least 24 hours and lactose was not fully utilised. The ultimate substrate consumption led to an intensive lovastatin biosynthesis (about 90 mg LOV l^{-1}) in the shake flasks, while in the bioreactor only about 11 mg LOV l^{-1} was obtained.
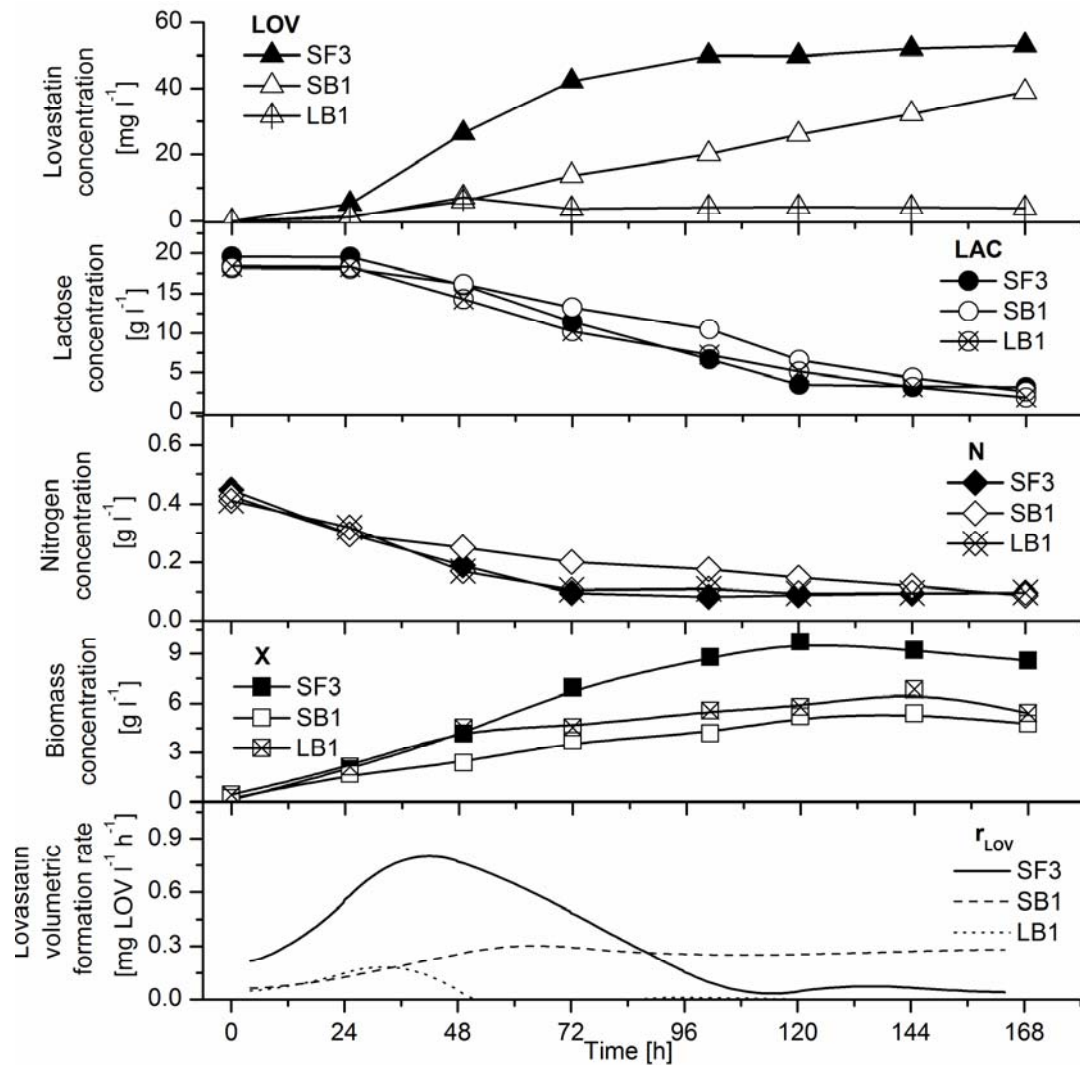
Fig. 2. Lovastatin, lactose, nitrogen and biomass concentration and lovastatin volumetric formation rate in the runs *SF3*, *SB1* and *LB1*; Nitrogen source: $c_{YE,0}=4$ g l^{-1} for all runs. Data for $c_{LAC,0}=20$ g l^{-1} in the shake flasks come from Bizukojć and Ledakowicz, (2007a). Data for $c_{LAC,0}=20$ g l^{-1} in the small stirred-tank bioreactor come from Bizukojć and Ledakowicz, (2008). Processes in the bioreactors without *pH* and *pO₂* control; aeration *SB1*: *vvm*=0.308 l$_{air}$ min^{-1} l^{-1}, *LB1*: *vvm*=0.3 l$_{air}$ min^{-1} l^{-1}

It is necessary to answer the question, what the reason of this phenomenon was, i.e. what influenced such a slow utilisation of substrates in the bioreactor and why the substrates in the shake flask were completely consumed. In this case the hypothesis about insufficient bioreactor aeration is more convincing, although a drastic oxygen limitation was still not observed as pO_2 was around 10% (Fig. 3). The researchers who conducted lovastatin biosynthesis in bioreactors claimed that there were several factors that may have played an important role in these processes (Bizukojć and Ledakowicz, 2008; Casas Lopez et al, 2004; Lai et al., 2005; Rodriguez Porcel et al., 2006). Among these factors the initial concentration of nitrogen sources, *pH* control and *pO₂* control were mentioned. Especially, *pH* curve is worth further analysis as it changes to a great extent within the cultivation time. One observes the maximum of about 7.6 at about 24 hour and a further decrease down to 6.4 at the end of the run. Thus, all these factors mentioned above are going to be studied further.
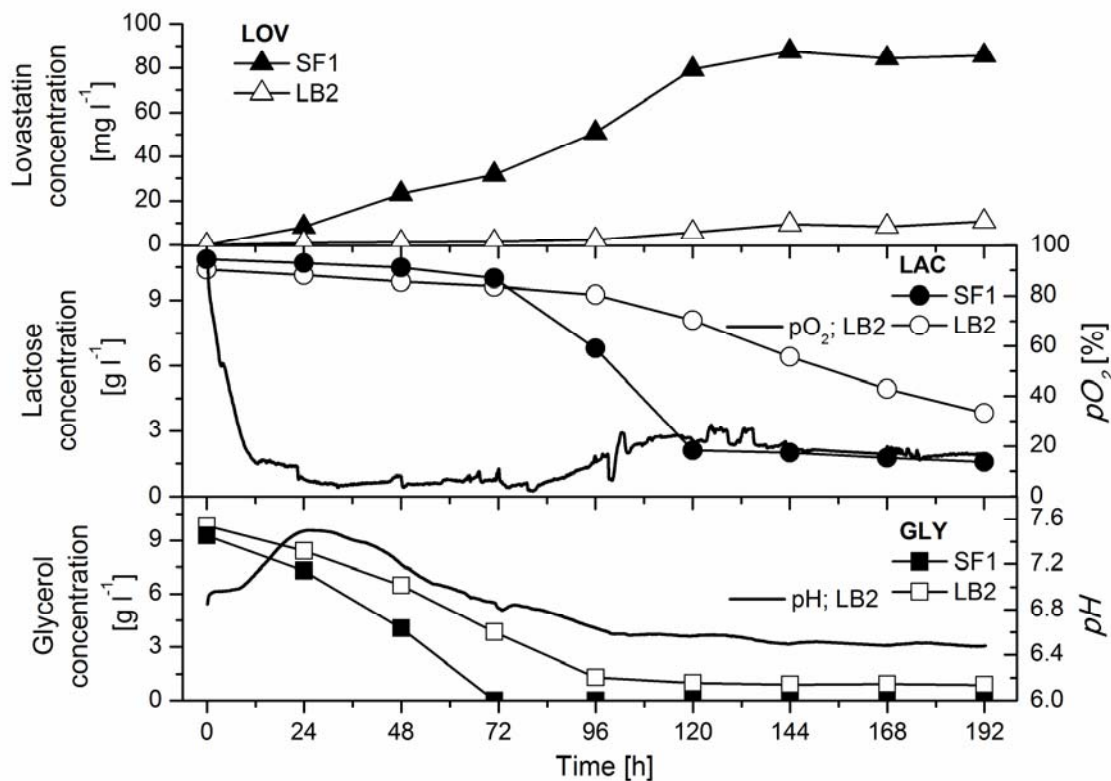
Fig. 3. Lovastatin, lactose and glycerol concentration obtained on the mixture of lactose and glycerol in the shake flasks and in the 5.3-litre stirred-tank bioreactor; $c_{LAC,0}$=10 g l^{-1}, $c_{GLY,0}$=10 g l^{-1}, $c_{YE,0}$=4 g l^{-1}. Process in bioreactor without pH and pO_2 control; Aeration: vvm=0.283 l$_{air}$ min^{-1} l^{-1}

3.3. Modification of initial nitrogen concentration

It was proved that an elevated organic nitrogen concentration inhibits lovastatin biosynthesis. For example, Casas Lopez et al. (2003) showed that the deficiency of nitrogen improved lovastatin titre. It was also confirmed by Bizukojć and Ledakowicz (2007a). However, all these results were obtained from shake flasks, not bioreactors.

Heretofore, processes have been presented, in which the initial nitrogen concentration was equal to 4 g l^{-1}. However, it is known from previous reports that this initial nitrogen concentration was optimal only in the runs performed in shake flasks (Bizukojć and Ledakowicz, 2007a), because in a 1.95-litre bioreactor the initial yeast extract concentration of 2 g l^{-1} was more favourable for lovastatin production (Bizukojć and Ledakowicz, 2008). Thus, the experiment in the 5.3-litre bioreactor with the use of the initial nitrogen concentration equal to 2 g l^{-1} and two carbon substrates (the best lactose and glycerol composition upon previous experiments) was conducted. In Fig. 4 the evolution curves of lovastatin, nitrogen, lactose, glycerol and biomass for the runs in both sizes of the stirred-tank bioreactors with the use of $c_{YE,0}$=2 g l^{-1} are presented.

Despite the fact that in the 5.3-litre bioreactor even two carbon sources were used, which should theoretically improve lovastatin production, the obtained lovastatin titres occurred to be worse than in the 1.95-litre bioreactor (SB2). So the application of a deficient amount of organic nitrogen in the larger stirred-tank bioreactor was not as encouraging as in the smaller one, despite the favourable carbon source sources mixture. The faster nitrogen uptake rate for the first 24 hours in the 5.3-litre bioreactor compared with the run in the 1.95-litre bioreactor was observed and this fact is probably connected with glycerol consumption as this substrate is faster assimilated by *A. terreus* than lactose (Bizukojć

and Pecyna, 2011). What is more, in the run *LB3* lactose consumption was not observed at all and it was not connected with oxygen deficiency (Fig. 4) but rather with the lack of organic nitrogen. It must be also mentioned here again that lactose and glycerol are equivalent substrates with regard to the amount of elemental carbon (Bizukojć and Pecyna, 2011).

To sum up, the decrease of the initial nitrogen concentration in the 5.3-litre bioreactor from 4 to 2 g l^{-1} did not improve lovastatin production. Additionally, analysing biomass concentration curves, slower biomass growth during first 48 hours in the run *LB3* was observed. Nevertheless, the biomass curves did not differ much. Still biomass concentration in the idiophase was at least by 30% lower than in the runs with 4 g of yeast extract per litre (compare Fig. 2 and 4). In the runs *SB3* and *LB2* the evolution of (+)-geodin was also analysed (data not shown). Enhanced biosynthesis of this metabolite in the large bioreactor (*LB3*) was observed and the amount of (+)-geodin in the broth exceeded 200 mg l^{-1}. The presence of glycerol in the medium probably had a significant influence on this results, which was mentioned in the previous section.
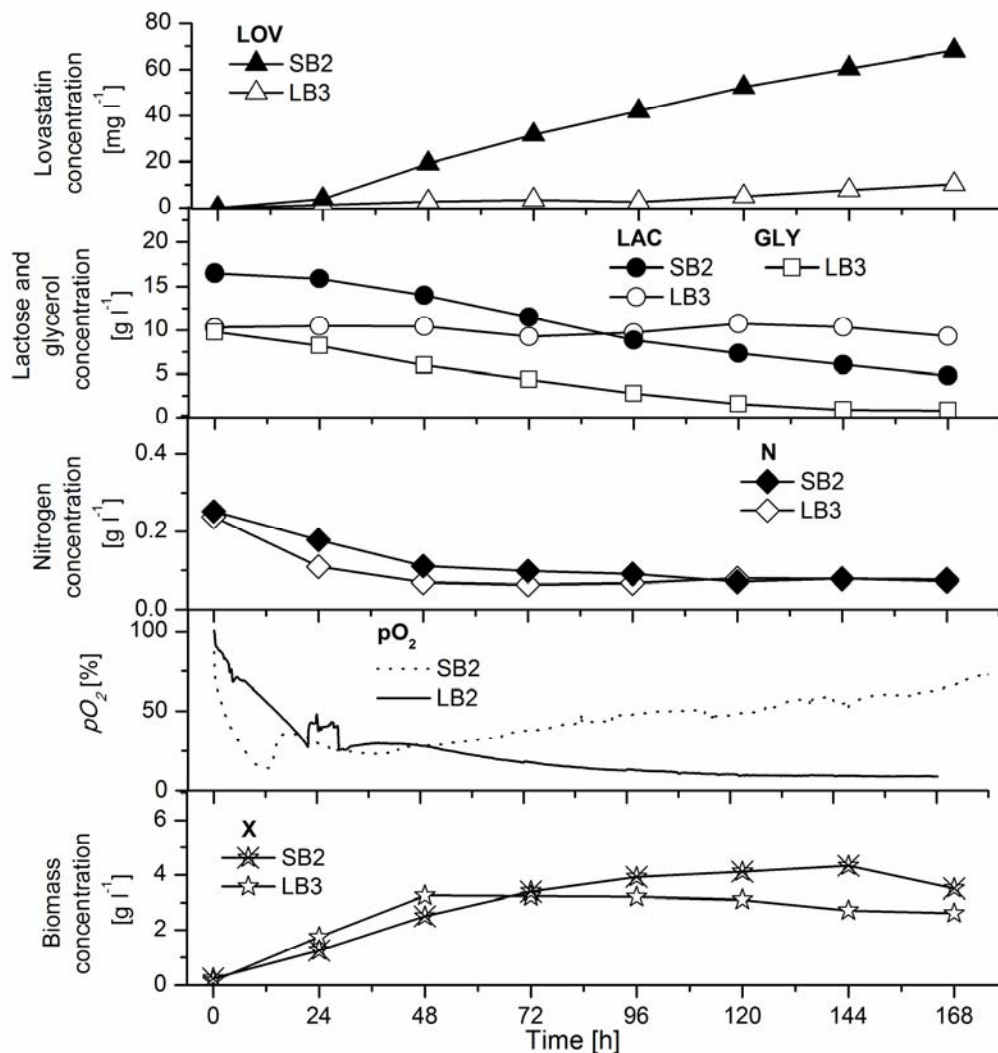

Fig. 4. Comparison of lovastatin, nitrogen, lactose, glycerol, biomass and dissolved oxygen concentrations for the runs *LB3* and *SB2*. Data for *SB2* come from Bizukojć and Ledakowicz (2008); $c_{LAC,0}$=20 g l^{-1}, vvm=0.308 l$_{air}$ min^{-1} l^{-1}, *LB3*: $c_{LAC,0}$=10 g l^{-1}, $c_{GLY,0}$=10 g l^{-1}, vvm=0.282 l$_{air}$ min^{-1} l^{-1}; runs without *pH* and *pO$_2$* control; Nitrogen source $c_{YE,0}$=2 g l^{-1}

3.4. Application of the simultaneous pH and pO₂ control

A study on the influence of *pH* control on lovastatin biosynthesis by *A. terreus* ATCC 20542 was previously conducted by Lai et al. (2005). In their experiments, 1 N HCl or 1 N NaOH solution were used to *pH* adjustment at three different levels i.e. 5.5, 6.5 and 7.5. This way Lai et al. (2005) concluded that *pH* control caused a decrease of lovastatin production and biomass concentration. They suggested that unsuitable *pH* could cause the activation of the enzymes that destroy the structure of lovastatin. At the same time these authors did not provide data on the impact of *pH* on (+)-geodin biosynthesis.

Other results of *pH* control in lovastatin biosynthesis were obtained by Bizukojć and Ledakowicz (2008) in the experiments in a 1.95-litre bioreactor. That *pH* level was controlled at 7.6 or 7.8 by an addition of sodium and potassium bicarbonate solution. The motivation for this action was the fact that several reactions of primary metabolism of *A. terreus* are dependent on bicarbonate ions concentration, including malonyl-CoA formation (Bizukojć and Ledakowicz, 2008). Malonyl-CoA is the substrate for polyketide chain elongation and used by both lovastatin nonaketide synthase and lovastatin diketide synthase. (Kennedy et al., 1999). The application of *pH* control also significantly decreased (+)-geodin concentration in the broth (Bizukojć and Ledakowicz, 2008).

Another factor influencing lovastatin formation was the concentration of dissolved oxygen in the medium. Casas Lopez at al. (2004) conducted experiments in a 5-litre bioreactor, in which aeration rate equal to 1 l_{air} min l^{-1} was applied. Additionally, aeration gas was oxygen-enriched. This treatment resulted in the increase of lovastatin production from 20 to 80 mg LOV l^{-1}. Rodriguez Porcel et al. (2006), also proved that the control of dissolved oxygen on the high level (400%) in the broth was necessary for attaining suitable lovastatin production in the slurry bioreactor. Lai et al. (2005) received the best results, when dissolved oxygen was controlled at the level of 20%. Bizukojć and Ledakowicz (2008), contradicted the result presented earlier, proving that a lower *vvm* at the level of about 0.3 l_{air} min^{-1} l^{-1} was more optimal for lovastatin biosynthesis. What is more, a high *vvm* equal to 1 l_{air} min^{-1} l^{-1} led to an efficient (+)-geodin formation (Bizukojć and Ledakowicz, 2008).

The actions described in the previous sections did not assure acceptable lovastatin titres in the 5.3 litre bioreactor and the aforementioned premises are sufficient to verify the simultaneous impact of *pH* and *pO₂* control in lovastatin biosynthesis in this bioreactor using glycerol and lactose as carbon sources.

At first, the experiments with (*SF/A2*) and without (*SF/A1*) bicarbonate solution addition in the shake flasks were performed and their results being the reference to the bioreactor runs, are shown in Fig. 5a

It is clearly seen that a satisfactory lovastatin concentration (77.3 mg LOV l^{-1}) was only obtained in the run *SF/A1* without bicarbonates addition. The appreciable increase in lovastatin concentration from 27.6 to 67.3 mg LOV l^{-1} between 96 and 120 hour took place. At the same time the levels of *pH*, after an initial increase, dropped from 6.67 to 6.35. In this run a decrease of *pH* in the late idiophase contributed to an increase of (+)-geodin formation compared to the run *SF/A2* and an addition of bicarbonates solution that increased *pH* in run *SF/A2* led to a significant decrease of the secretion of this by-product. It was in agreement with the previous observations of Bizukojć and Ledakowicz (2008). Thus, it is easy to notice that in the processes conducted in the shake flasks *pH* control, actually an addition of bicarbonates solution was not advisable with regard to lovastatin biosynthesis but it was a suitable method to decrease (+)-geodin concentration in the broth. The consecutive step was to check if the application of *pH* control would lead to an improvement of lovastatin production in the 5.3-litre bioreactor, bearing in mind disappointing results from the shake flask culture and previous encouraging results obtained in the 1.95-litre bioreactor (Bizukojć and Ledakowicz, 2008).
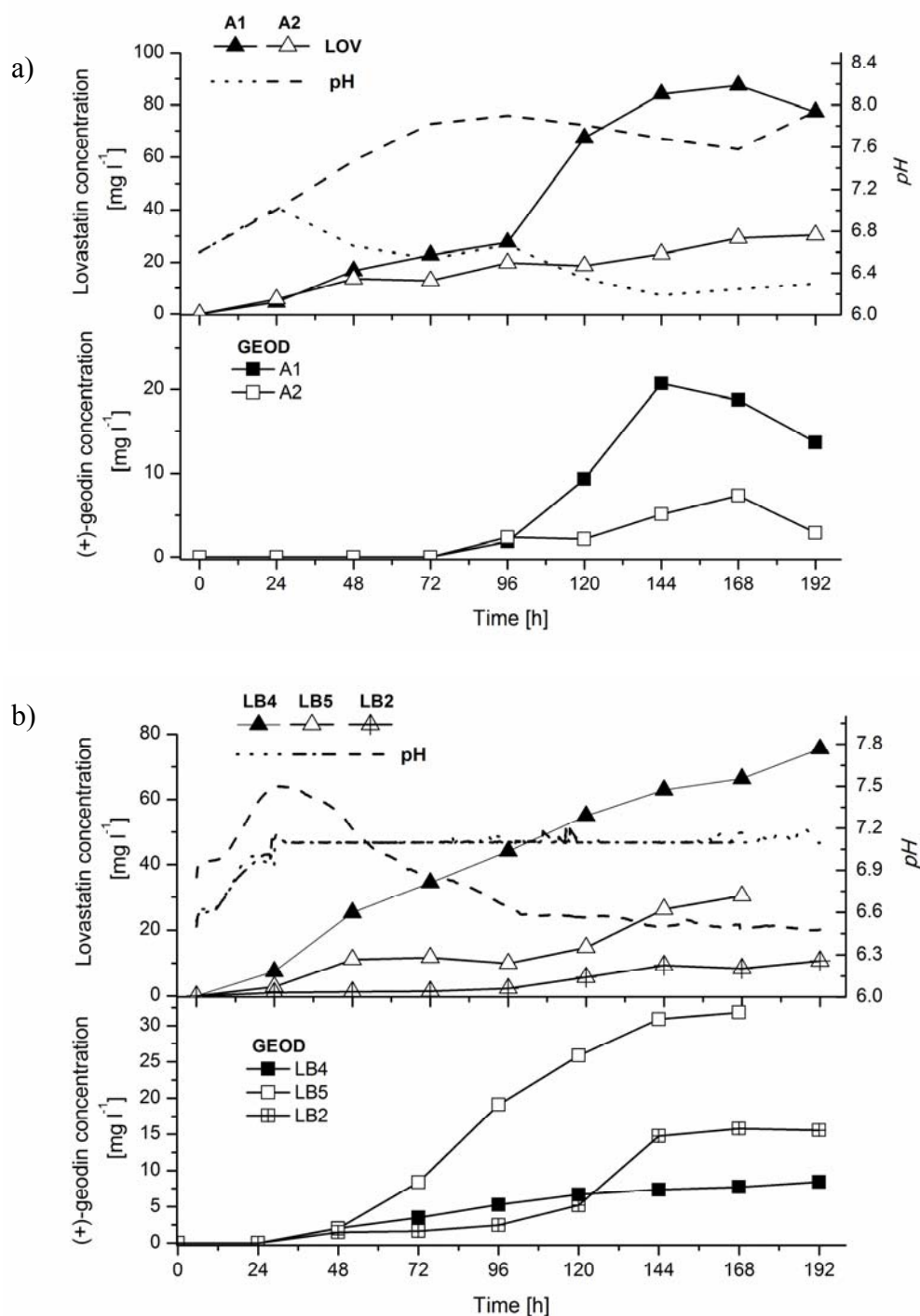
Fig. 5. Comparison of lovastatin and (+)-geodin concentration, influence of *pH* and *pO₂* control: (a) processes in the shake flasks without *SF/A1* and with *SF/A2* with *pH* control; (b) processes in the bioreactor run *LB4 pH* controlled with NaHCO₃ and KHCO₃, run *LB5 pH* controlled with NaOH, run *LB2* without *pH* control; runs *SF/A1*, *SF/A2*, *LB4*, *LB5*, *LB2* ($c_{LAC,0}$=10 g l⁻¹, $c_{GLY,0}$=10 g l⁻¹, $c_{YE,0}$=4 g l⁻¹), runs *LB4* and *LB5*: *pO₂* control at 20%, run *LB2* aeration at *vvm*=0.283 l$_{air}$ min⁻¹ l⁻¹

In Fig. 5b the results from the run *LB4* (*pH* controlled at 7.15 with a solution of NaHCO₃ and KHCO₃) and from *LB5* (*pH* controlled at 7.15 with a solution of NaOH) were compared to the earlier presented run *LB2* (*pH*-uncontrolled). It should be noted that in the runs *LB4* and *LB5* also *pO₂* control at 20% saturation was applied for the reasons described further in this section. In the runs conducted in the 5.3-litre bioreactor the application of the simultaneous *pH* and *pO₂* control led to a significant increase of lovastatin formation (75.5 mg LOV l⁻¹). This result was now closer to the titre obtained in the shake

flask run (*SF/A1)* but without *pH* control (87.4 mg LOV l^{-1}) than in the other bioreactor runs described here. Thus, higher *pH* seems to be favourable in the bioreactor, not in the shake flasks. But what is the most important thing, it was also observed that the use of bicarbonate ions was most favourable for lovastatin formation by *A. terreus* ATCC 20542, because in the run *LB5*, in which the solution of NaOH was used the enhancement of lovastatin production was much weaker compared to the run *LB4*. Thus, it is not only the effect of *pH* but of inorganic carbon in the form of bicarbonate that influences lovastatin formation. Additionally, bicarbonate ions to a greater extent than hydroxide ions contributed to the inhibition of (+)-geodin formation. Now, it became understandable why Lai et al. (2005) did not recommend *pH* control, as they used NaOH and HCl solutions for this purpose.

It was found earlier (Bizukojć and Ledakowicz, 2008) that an increase of aeration in the 1.95-litre bioreactor rate and as a consequence of oxygen saturation in the system led to efficient (+)-geodin formation, whose titre even exceeded 100 mg GEOD l^{-1}, and worse lovastatin formation. So at the first glance it seemed not to be the right action to increase either aeration rate or oxygen saturation in the 5.3-litre bioreactor. In the 1.95-litre bioreactor the optimal *vvm* was equal to about 0.3 l$_{air}$ min^{-1} l^{-1}. However, it is seen in Fig. 6 that at a low *vvm* substrate uptake rate deteriorated in the 5.3-litre bioreactor and was even lower than in the shake flasks. So it was decided to hold oxygen saturation at 20% level by increasing the air flow rate and rotary speed of the impeller in this bioreactor. In this way the range of *vvm* varied from 0.282 to 0.998 l$_{air}$ min^{-1} l^{-1} dependent on the time of the process (Fig. 6).
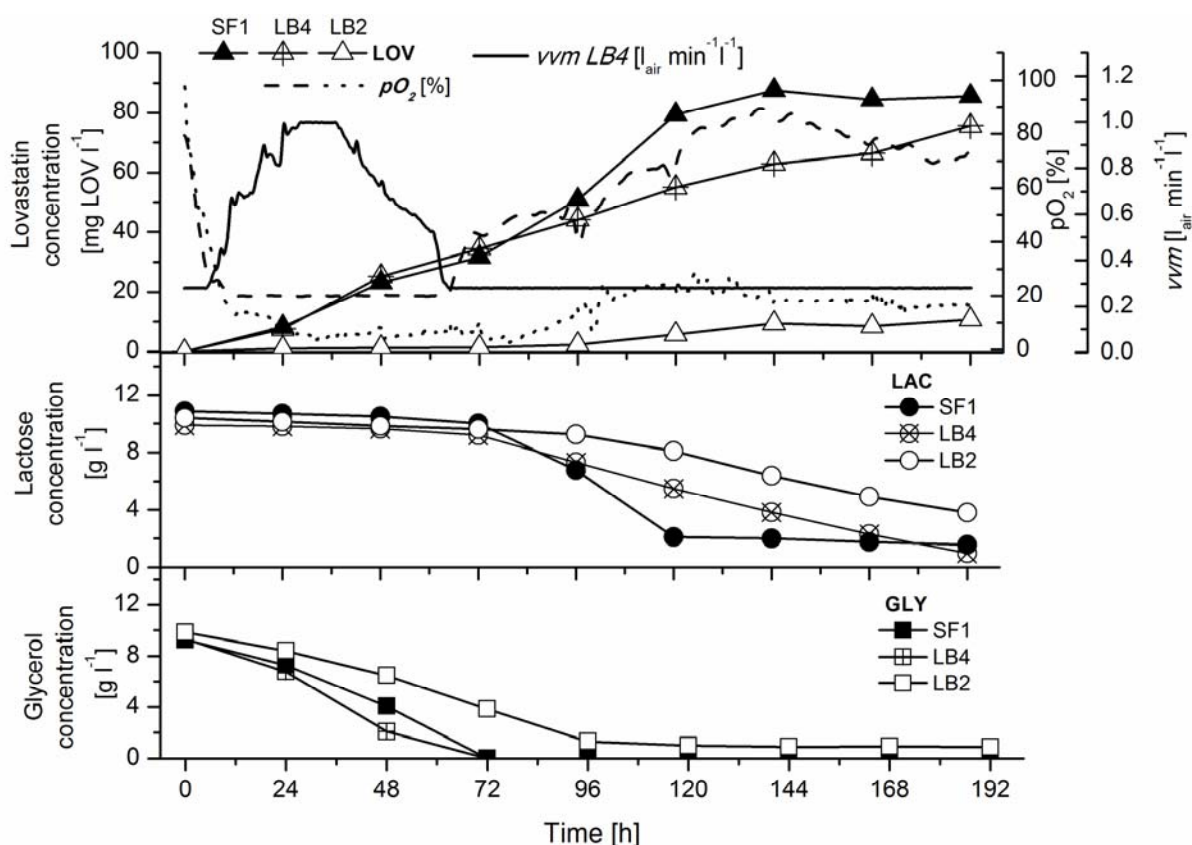

Fig. 6. Influence of *pO₂* control on the formation of lovastatin and on the lactose and glycerol uptake rates in runs *SF1, LB2, LB4*; *pO₂* control at 20% and *pH* control, *LB2*; *vvm*=0.283 l$_{air}$ min^{-1} l^{-1}

First of all, this action did not lead to elevated (+)-geodin levels, due to simultaneous *pH* control. Secondly, as a result of *pO₂* control at the level of 20% in the run *LB4* up to 72 hour a similar uptake rate of glycerol compared with the run *SF/A1* was observed. It was much higher than in the run *LB2* without *pO₂* and *pH* control. What is more, the same amount of lovastatin for *SF/A1* and *LB4* within

this period of time was obtained (Fig. 6). Unfortunately, later despite high concentration of dissolved oxygen in the run *LB4* the consumption of lactose was still lower compared with the run *SF1* and lovastatin formation slowed down. However, this is the evidence that in the bioreactor systems the application of simultaneous *pH* and *pO₂* control is necessary in order to increase lovastatin production and to follow the course of the process as in the efficient shake flask experiment.

To sum up, the discussion above indicates that a simple transfer of the medium for lovastatin production from the efficient shake flask culture to the bioreactor leads to the worsening of the process with regard to lovastatin production. Therefore, several additional measures, namely *pO₂* control, which is quite obvious, and *pH* control with bicarbonate solution, which is far more surprising and less obvious, must be taken to achieve at least similar results in the bioreactor compared to those in the shake flasks.

4. CONCLUSIONS

The drawn conclusions are going to give some practical guidance to the problem of how to transfer lovastatin production from the shake flasks to the bioreactor. Thus, on the basis of the presented results it can be said that:

- Using the same type and concentration of carbon and nitrogen sources in the bioreactor, considerably lower lovastatin titres are obtained compared with the similar processes conducted in the shake flasks.

- A low initial nitrogen concentration (2 g l^{-1}) may increase lovastatin formation but not always as it was observed only in the 1.95-litre stirred tank bioreactor. On the other hand it may also lead to a decrease of substrate uptake rate and worse biomass formation aggravating lovastatin production too, which was observed in the 1.95-litre bioreactor.

- The application of *pH* control using bicarbonates solution in the processes conducted in the shake flasks does not lead to the enhancement of lovastatin production, although this control slightly decreases the formation of the by-product, (+)-geodin. When *pH* and *pO₂* control are used simultaneously in the 5.3-litre bioreactor, a satisfactory lovastatin titre is obtained; even seven times higher lovastatin concentration compared with the bioreactor runs without pH and *pO₂* control. It is definitely important to control pH at a mild alkaline level slightly above 7 with bicarbonates solution, as the use of sodium hydroxide does not give such good effects. Thus, not only pH but the effect of bicarbonate ions is favourable for lovastatin production.

- A controlled level of dissolved oxygen in the medium for the run in the 5.3-litre bioreactor is required for the efficient utilisation of the substrates, which somewhat enhances lovastatin production.

REFERENCES

Bizukojć M., Ledakowicz S., 2007a. A macrokinetic modelling of the biosynthesis of lovastatin by *Aspergillus terreus*. *J. Biotechnol.*, 130, 422–435. DOI: 10.1016/j.biotec.2007.05.007.

Bizukojć M., Ledakowicz S., 2007b. Simultaneous biosynthesis of (+)-geodin by a lovastatin-producing fungus *Aspergillus terreus*. *J. Biotechnol.*, 132, 453-460. DOI: 10.1016/j.jbiotec.2006.06.017.

Bizukojć M., Ledakowicz S., 2008. Biosynthesis of lovastatin and (+)-geodin by *Aspergillus terreus* in batch and fed-batch culture in the stirred tank bioreactor. *Biochem. Eng.,* 42, 198-207. DOI: 10.1016/j.bej.2008.06.022.

Bizukojć M., Pecyna M., 2011. Lovastatin and (+)-geodin formation by *Aspergillus terreus* ATCC 20542 in a batch culture with the simultaneous use of lactose and glycerol as carbon sources. *Eng. Life Sci.,* 11, 272-282. DOI: 10.1002/elsc.201000179.

Casas Lopez J.L., Sanchez Perez J.A., Fernandez Sevilla J.M., Acien Fernandez F.G., Molina Grima E., Chisti Y., 2003. Production of lovastatin by *Aspergillus terreus:* effects of C:N ratio and the principal nutrients on growth and metabolite production. *Enzyme Microb. Technol*., 33, 270-277. DOI: 10.1016/S0141-0229(03)00130-3.

Casas Lopez J.L., Sanchez Perez J.A., Fernandez Sevilla J.M., Acien Fernandez F.G., Molina Grima E., Chisti Y., 2004. Fermentation optimization for the production of lovastatin by *Aspergillus terreus*; use of response surface methodology. *J. Chem. Technol. Biotechnol.,* 79, 1119–1126. DOI: 10.1002/jctb.1100.

Casas Lopez J.L., Sanchez Perez J.A., Fernandez Sevilla J.M., Rodriguez Porcel E.M., Chisti Y., 2005. Pellet morphology, culture rheology and lovastatin production in cultures of *Aspergillus terreus*. *J. Biotechnol.,* 116, 61-77. DOI: 10.1016/j.jbiotec.2004.10.005.

Hajjaj H., Niederberger P., Duboc P., 2001. Lovastatin biosynthesis by *Aspergillus terreus* in a chemically defined medium. *Appl. Environ. Microbiol.,* 67, 2596-2602. DOI: 10.1128/AEM.67.6.2596-2602.2001.

Kennedy J., Auclair K., Kendrew S.G., Park C., Vederas J., Hutchinson C.R., 1999. Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science,* 284, 1368-1372. DOI: 10.1126/science.284.5418.1368.

Kumar M.S., Jana S.K., Senthil V., Shashanka V., Kumar S.V. Sadhukhan A.K., 2000. Repeated fed-batch process for improving lovastatin production. *Proc. Biochem.,* 36, 363-368. DOI: 10.1016/S0032-9592(00)00222-3.

Lai L-S.T., Pan C-C., Tzeng B-K., 2002. Medium optimization for lovastatin production by *Aspergillus terreus* in submerged cultures. *J. Chin. Inst. Chem. Engrs*., 33, 517- 527.

Lai L-ST., Tsai T-H, Wang T.C., Cheng T.Y., 2005. The influence of culturing environments on lovastatin production by *Aspergillus terreus* in submerged cultures. *Enz. Microb. Technol.,* 36, 737-748. DOI: 10.1016/j.enzmictec.2004.12.021.

Manzoni M., Rollini M., Bergomi S., Cavazzoni V., 1998 . Production of statinsby filamenous fungi. *Biotechnol. Lett.,* 21, 253-257. DOI: 10.1023/A:1005495714248.

Rodriguez Porcel E.M., Casas López J.L., Sanchez Pérez J.A., Fernández Sevilla J.M., Garcia Sánchez J.L., Chisti Y., 2006. *Aspergillus terreus* broth rheology, oxygen transfer, and lovastatin production in a gas-agitated slurry reactor. *Ind. Eng. Chem. Res*., 45, 4837-4843. DOI: 10.1021/ie0600801.