Chemical and Process Engineering 2012, 33 (4), 651-665
DOI: 10.2478/v10176-012-0055-5

KINETIC MODELLING OF LOVASTATIN BIOSYNTHESIS BY *ASPERGILLUS TERREUS* CULTIVATED ON LACTOSE AND GLYCEROL AS CARBON SOURCES

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A kinetic model to describe lovastatin biosynthesis by *Aspergillus terreus* ATCC 20542 in a batch culture with the simultaneous use of lactose and glycerol as carbon sources was developed. In order to do this the kinetics of the process was first studied. Then, the model consisting of five ordinary differential equations to balance lactose, glycerol, organic nitrogen, lovastatin and biomass was proposed. A set of batch experiments with a varying lactose to glycerol ratio was used to finally establish the form of this model and find its parameters. The parameters were either directly determined from the experimental data (maximum biomass specific growth rate, yield coefficients) or identified with the use of the optimisation software. In the next step the model was verified with the use of the independent sets of data obtained from the bioreactor cultivations. In the end the parameters of the model were thoroughly discussed with regard to their biological sense. The fit of the model to the experimental data proved to be satisfactory and gave a new insight to develop various strategies of cultivation of *A. terreus* with the use of two substrates.

Keywords: lovastatin, multi-substrate kinetics, modelling, batch bioreactor

1. INTRODUCTION

The natural cholesterol lowering agent called lovastatin is a polyketide metabolite secreted by such filamentous fungi as *Aspergillus terreus* and *Monascus ruber*. This compound acts in the human organism as a competitive inhibitor of (S)-3-hydroxymethylglutaryl-CoA reductase, blocking endogenous cholesterol formation at the early stage.

Since its discovery, lovastatin biosynthesis has been widely investigated. Researchers took into consideration such factors as a type of carbon and nitrogen sources and their concentrations, oxygen saturation, aeration rate, pH and fungal morphology (Bizukojć and Ledakowicz, 2010; Casas López et al., 2003, 2005; Lai et al., 2005; Rodríguez Porcel et al., 2006). On the basis of these studies it can be concluded that such carbon substrates as lactose or glycerol were most suitable for lovastatin production (Bizukojć and Pecyna, 2011; Casas López et al., 2003,). What is even more important, even better results were obtained when two or more carbon sources were applied at the same time (Bizukojć and Pecyna, 2011; Hajjaj et al., 2001; Kumar et al., 2000). With regard to nitrogen sources organic nitrogen such as yeast extract, corn steep liquor, soybean flour or casein peptone are preferred for lovastatin production. Nevertheless, their concentration must not be too high due to the inhibition of product formation caused by organic nitrogen (Bizukojć and Ledakowicz, 2007a; Casas López et al., 2003).

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Taking the kinetics of product formation into account, lovastatin is formed in the trophophase and its formation is to a high extent growth associated, especially when an individual carbon source is used in the batch system At the same time lovastatin can be also produced in the idiophase, which is observed in the fed-batch culture being fed with a carbon substrate (Bizukojć and Ledakowicz, 2007). All in all, lovastatin production is claimed to be mixed growth-associated.

An important part of any research concerning production of a metabolite by a microorganism is the kinetic model of the process and so is the case with *A. terreus* and lovastatin production. In the literature, there are two kinetic models, which describe biomass growth and lovastatin biosynthesis by *A. terreus.* The first structured model was formulated by Liu et al., 2000 and was strongly based upon the previous models formulated for penicillin production by *Penicillium chrysogenum*. Whether this model suits well to describe lovastatin formation by *A. terreus* can be questionable, as it lacks nitrogen balance, which is an important variable influencing lovastatin biosynthesis (actually its inhibitor). Furthermore, the usage of the parameters previously determined for *P. chrysogenum* can be sometimes doubtful. And last but not least, in its structured part hyphal differentiation was assumed to be exactly the same as for *P. chrysogenum*. The problem is that *P. chrysogenum* is characterised by a different morphology, in most cases dispersed, and even if it forms pellets, the mechanism of pellet formation in *Penicilli* is different than that in *Aspergilli* (Metz and Kossen, 1977). *A. terreus* almost always grows in the form of macroscopic pellets (Bizukojć and Ledakowicz, 2010; Rodríguez Porcel et al., 2006).

In the recently published unstructured model by Bizukojć and Ledakowicz (2007) a different modelling approach was used. These authors formulated a kinetic model for lovastatin production in batch and in fed-batch processes on lactose as the sole carbon source. This model was based upon on their own experimental data only. Most of model parameters occurred to be constant independent of process conditions. The experiments, upon which this model was formulated, were conducted in a wide range of lactose from 5 to 40 g l^{-1} and yeast extract (organic nitrogen) from 2 to 12 g l^{-1} concentrations. Thus, it described a system with an individual carbon source (lactose) and individual nitrogen source (yeast extract). It rather imperfectly described biomass growth due to the reasons that are going to be further presented in this work.

As mentioned above, two carbon sources are more favourable for lovastatin production than individual ones. Thus, a need to widen and supplement the previous model by Bizukojć and Ledakowicz (2007) occurred. Therefore, the main purpose of this study was to formulate the kinetic model of lovastatin biosynthesis by *A. terreus* ATCC 20542 on lactose and glycerol being utilised simultaneously. The formulation of the model is going to be performed upon the data from the batch shake flask culture for the various initial lactose and glycerol ratios. Upon these experiments the parameters of the model are to be found, while the data from the bioreactor processes shall serve as the verification of the model.

2. MATERIALS AND METHODS

2.1. Strain and media

The strain *Aspergillus terreus* ATCC 20542 was used in the experiments of lovastatin biosynthesis. They were conducted in shake flasks of 150 ml working volume and in a stirred-tank bioreactor of 5.3-litre working volume, both 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 200 min^{-1}. The control of pH was performed with a solution of sodium and potassium bicarbonate and kept at the levels close to 7. 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 $MgSO_4 \cdot 7\ H_2O$: 0.52 g l^{-1}, sodium chloride NaCl: 0.4 g l^{-1}, zinc sulphate $ZnSO_4 \cdot 7\ H_2O$: 1 mg l^{-1}, ferric nitrate $Fe(NO)_3 \cdot 9\ H_2O$: 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 borate $Na_2B_4O_7 \cdot 10\ H_2O$: 100 mg l^{-1}, manganese chloride $MnCl_2$: 50 mg l^{-1} sodium molybdate $Na_2MoO_4 \cdot 2H_2O$: 50 mg l^{-1} and copper sulphate $CuSO_4 \cdot 5H_2O$: 250 mg l^{-1} Yeast extract (BD, USA) was used as the nitrogen source at the concentration of 4 g l^{-1} (8 g l^{-1} in the preculture) (Bizukojć and Ledakowicz, 2007). In shake flasks runs *AB1*, *AB2*, *AB3* the initial glycerol concentration was 5, 10 and 15 g l^{-1}. In bioreactors runs *BB1*, *BB2*, *BB3* the same glycerol concentration equal to 10 g l^{-1} was applied. In all these processes the initial lactose concentration was 10 g l^{-1}. The repeatability of the shake flask culture processes was tested previously (Bizukojć et al., 2007).

Additionally, shake flask runs to determine biomass maximum specific growth rate and study the initial stages of biomass growth were designated as *ZA*, *ZB* and *ZC*. In run *ZA* the initial lactose concentration was 25 g l^{-1}, in run *ZB* the initial glycerol concentration was 25 g l^{-1}, and in run *ZC* both lactose and glycerol concentrations were 10 g l^{-1}.

2.2. Analytical methods

Lovastatin was analysed with UPLC® (Waters, USA) in a RP18 1.7 µm (2×150 mm) column, at the flow rate 0.200 ml min^{-1}. The gradient elution was made with H_2O-CH_3CN (from 60:40 to 40:60 v/v) solutions modified with 1% HCOOH. The temperature of the column was 40°C. A photodiode array detector at λ=238 nm was used. Lactose and glycerol were determined isocratically in an amide column 1.7 µm (2.1×100 mm) using the eluent at the flow rate 0.290 ml min^{-1} containing 75% CH_3CN in water with 2% triethylamine as a modifier, at 35°C. Biomass was dried at 105°C to a constant weight. Organic nitrogen and carbon were 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. Oxygen profiles in the pellets were measured with the use of an oxygen microprobe with a 10 µm tip controlled by a computerised micromanipulator (Unisense, Denmark).

2.3. Modelling tools

Some model parameters were initially directly determined from experimental data. These were yield coefficients $Y_{X/LAC}$, $Y_{X/N}$, $Y_{X/GLC}$, $Y_{LOV/LAC}$, $Y_{LOV/GLC}$ and maximum specific biomass growth rate μ_{max}. The values of yield coefficients were treated as the starting values for the optimisation algorithm. A few of them were slightly tuned while being optimised.

All the other parameters of the model were estimated with Easy-fit 4.21 software (© Klaus Schittkowski (2007), University of Bayreuth, Germany) using the modified quasi-Newton least squares optimisation. In order to solve a system of ordinary differential equations (ODE) the implicit 5th order Runge-Kutta method for stiff ODEs was used.

3. RESULTS AND DISCUSSION

3.1. Lovastatin formation kinetics at various lactose to glycerol ratios

It was previously proved that the application of lactose and glycerol for lovastatin biosynthesis by

A. terreus both in shake flasks and bioreactor led to an increase of lovastatin titre compared to the experiments, in which only one of these substrates was used. This phenomenon was the result of the interaction of these two carbon sources, which prolonged fungal viability as well as the increase in product formation volumetric rate (Pawlak and Bizukojć, 2012). Both substrates played an important role in the mixed growth associated lovastatin formation and the biosynthesis of this metabolite was strongly dependent on both lactose and glycerol. The association of lovastatin formation with lactose, glycerol, nitrogen utilisation rates and biomass growth rate for run *AB2* is shown in Fig. 1.

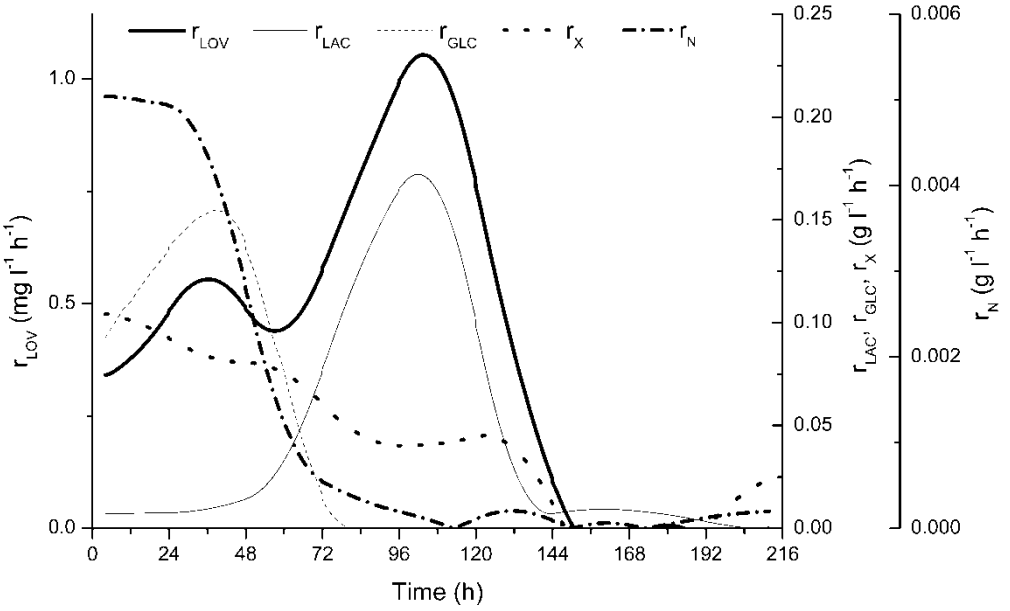

Fig. 1. Time changes of volumetric formation rate of lovastatin, volumetric biomass growth rate and volumetric uptake rates of lactose, glycerol and nitrogen in run *AB2*

The maximum values of biomass volumetric formation rate (r_X) occurred in the first 24 hours in this run, whereas lovastatin volumetric formation rate (r_{LOV}) had two maxima. The first maximum was observed at 36 hours of the run and was strictly connected with glycerol utilisation. The second maximum of r_{LOV} , which can be attributed to lactose assimilation, was observed at 100 hours of the run. Thus, these two maxima did not overlap in time. It is a typical feature of the system, in which two carbon substrates are consecutively utilised. It can be also noticed in this graph that the maximum of volumetric nitrogen uptake rate (r_N) occurred approximately at the same moment as the maximum values of r_X. This is an indirect evidence that biomass growth strongly depended on nitrogen source and it was nitrogen, which was probably the limiting substrate for biomass growth, not lactose or glycerol. This association was not observed for r_{LOV} and r_N, which indicated that lovastatin production and nitrogen uptake were not related directly. As long as nitrogen was taken up intensively lovastatin formation was not very high. It was also connected with the fact of the repression of lovastatin formation due to the elevated organic nitrogen level. At the same time organic nitrogen influenced lovastatin production through its effect on biomass growth. Too low biomass levels are not favourable for biosynthesis of lovastatin (Bizukojć and Ledakowicz, 2007; Pawlak and Bizukojć, 2012). It should be also noted that in this work the optimal nitrogen concentration both for the runs conducted in the shake flasks and in the bioreactor was used (4 g l^{-1}) to minimise the repression of lovastatin formation and not to lead at the same time to the deterioration of biomass growth due to lack of this nutrient.

The consecutive utilisation of substrates and biomass growth is also seen in Fig. 2, in which the time changes of lovastatin, lactose, glycerol and biomass concentrations in the runs *AB1*, *AB2* and *AB3* were shown.

As already suspected from Fig. 1, it was clear that glycerol was utilised as the first substrate and then subsequently lactose. As long as glycerol was present in the medium, lactose was not used by *A. terreus*, so glycerol strongly repressed lactose uptake. This phenomenon is characteristic for diauxic growth of biomass but, what is surprising, in biomass curves this diauxic behaviour was not observed at all. It was also previously noticed by Bizukojć and Pecyna (2011). Further, it will be shown that biomass growth was quite untypical, partially due to non-biological reasons. The next observation was that in runs *AB2* and *AB3*, in which lactose concentration decreased earlier to the level of about 1.5 g l^{-1} (at 120 hours) lovastatin production ceased. A strong association of lovastatin production with carbon substrate (lactose) uptake was confirmed again. The best lovastatin titres were obtained when carbon source (lactose) concentration was on a relatively high level until the end of the process (*AB3*).

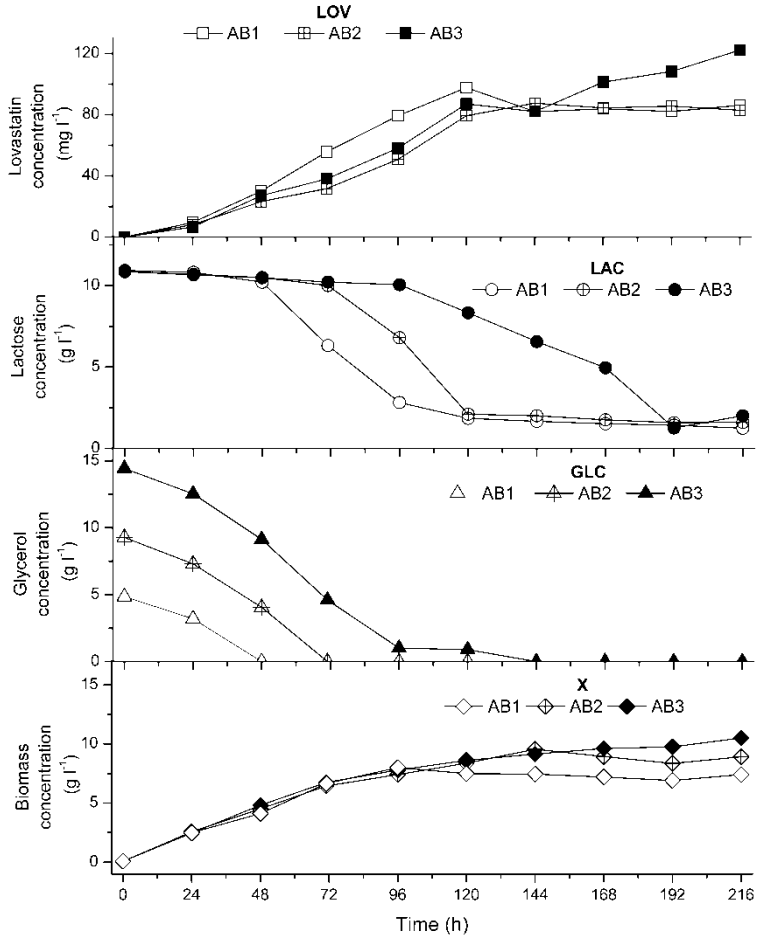

Fig. 2. Changes of lovastatin, lactose and glycerol concentration in runs *AB1*, *AB2*, *AB3*

Coming back to the untypical biomass growth of *A. terreus* during lovastatin biosynthesis, a more detailed study (more sampling points in time) concerning biomass formation within the first 24 hours of the run is presented in Fig. 3. It was already seen in Fig. 2 that the trophophase was not characterised by the exponential biomass growth, as one could normally expect, but by a linear growth. From Fig. 3 it can be concluded that *A. terreus* really grew exponentially with similar maximum biomass growth rates, irrespective of the medium composition (runs *ZA*, *ZB* and *ZC*), within the first 12 hours only. Maximum biomass specific growth (μ_{max}) was thereby equal to 0.12 h^{-1}. Then, one observed a much slower linear growth of biomass (compare Fig. 2 and 3). The reason for this phenomenon was that within these first twelve hours the diameter of fungal pellets did not exceed 500 μm and the further linear growth was caused by the fact that *A. terreus* evolved to the form of macroscopic pellets whose diameter was equal to 1-3 mm. As a result substrate availability may have been limited by mass transfer resistance.

Also it is seen in Fig. 3 that in the processes, in which glycerol as carbon sources was applied, a short lag-phase within the first three hours could be observed, because in preculture lactose, instead of glycerol, as the initial carbon source was always used and *A. terreus* had to adapt itself to the new substrate.

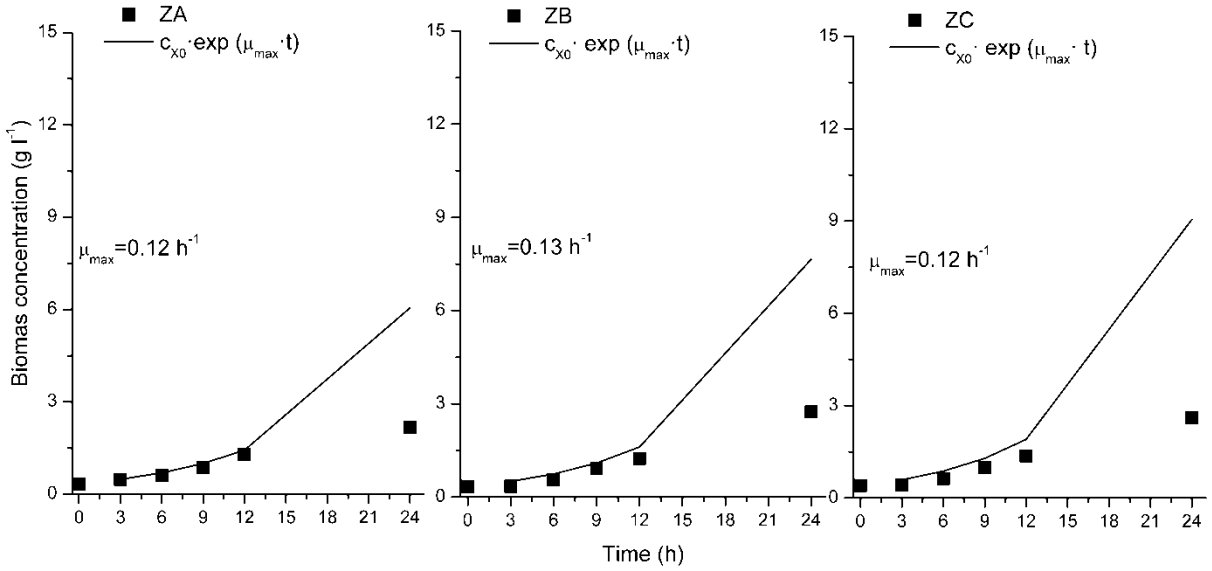

Fig. 3. Untypical biomass growth during lovastatin biosynthesis by *A. terreus* (runs *ZA*, *ZB*, *ZC*)

4. KINETIC MODEL FOR LOVASTATIN BIOSYNTHESIS ON A MIXTURE OF LACTOSE AND GLYCEROL

4.1. Main assumptions of the model

On the basis of the present results and few prerequisites from the previous work (Bizukojć and Ledakowicz, 2007) the following assumptions for the kinetic model of lovastatin biosynthesis by *A. terreus* can be pointed out.

- The mixture of lactose and glycerol of various ratios was used as the carbon sources. These substrates were utilised as the sole carbon sources. Amino acids from yeast extract in the medium were present but not used as carbon sources. Yeast extract was regarded as the sole organic nitrogen source. Thus, it produced three limiting substrates in the system: glycerol, lactose and organic nitrogen. As the initial concentration of the substrates was relatively high, constant values of yield coefficients were assumed and no maintenance was taken into account. Consequently, it was not necessary to determine maintenance coefficients, whose estimation upon the data from batch culture is troublesome and prone to the high error
- Not all nitrogen substrate from yeast extract was utilisable by *A. terreus*. So in order to simplify modelling, nitrogen concentration represents only this pool of nitrogen that was utilised. In all previous works it was observed that from 8 to 10 mg l^{-1} of nitrogen remained unused irrespective of carbon substrates used and all other factors (Bizukojć and Ledakowicz, 2007; Bizukojć and Pecyna 2011).
- Glycerol was always assimilated as the first substrate, then lactose. Therefore, the presence of glycerol in the medium repressed lactose utilisation, regardless of glycerol concentration.
- Lovastatin biosynthesis was mixed growth associated.
- Due to the repression mentioned in point 3 glycerol was utilised for lovastatin production mainly in the growth phase, while lactose in the late trophophase and in the idiophase.

- Contois model was used as a limiting term throughout the model. By trial-and-error method it was found that it better describes fungal systems than the Monod term. It is connected with high biomass concentration and its morphological form.
- Lovastatin formation was inhibited by organic nitrogen (Bizukojć and Ledakowicz, 2007).

4.2. Model equations

On the basis of the assumptions of this model the following equations were proposed. These equations (1-5) describe the balances of substrates i.e. lactose (c_{LAC}), glycerol (c_{GLC}) and organic nitrogen from yeast extract (c_N), the balance of product, namely lovastatin (c_{LOV}), and finally biomass (c_X).

$$\frac{dc_{LAC}}{dt} = -\frac{1}{Y_{X/LAC}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{X} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot \frac{K_{I,GLC,1}}{K_{I,GLC,1}+c_{GLC}} \cdot c_X - \\ -\frac{1}{Y_{LOV/LAC}} \cdot q_{\max}^{LOV} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{LOV} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLC}^{LOV} \cdot c_X} \cdot \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV}+c_N} \cdot \frac{K_{I,GLC,2}}{K_{I,GLC,2}+c_{GLC}} \cdot c_X \tag{1}$$

$$\frac{dc_{GLC}}{dt} = -\frac{1}{Y_{X/GLC}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{X} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot c_X - \\ -\frac{1}{Y_{LOV/GLC}} \cdot q_{\max}^{LOV} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{LOV} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLC}^{LOV} \cdot c_X} + \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV}+c_N} \cdot c_X \tag{2}$$

$$\frac{dc_N}{dt} = -\frac{1}{Y_{X/N}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{X} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLC}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot c_X \tag{3}$$

$$\frac{dc_{LOV}}{dt} = Y_{LOV/X} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{X} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot c_X + \\ +q_{\max}^{LOV} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{LOV} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLC}^{LOV} \cdot c_X} \cdot \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV}+c_N} \cdot c_X \tag{4}$$

$$\frac{dc_X}{dt} = \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{X} \cdot c_X} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLC}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot c_X \tag{5}$$

This model consists of five equations and there were 16 parameters to be identified. At the same time due to the presence of three limiting substrates the volumetric rates of each balanced metabolite are the mathematical product of three Contois limitation terms. This approach may lead to the falsified solutions, i.e. too low values of volumetric rates (left-hand sides of the equations). This problem of multi-substrate kinetics for Monod limitation terms was discussed by Nielsen (2006). He showed that multiplying any terms of a value lower than one (these are the values of any limitation terms, irrespective of the fact, whether they are Monod or Contois limitation terms), one finally gets lower and lower results of multiplication. Therefore, in the next step some limitation terms were removed, i.e. their values were set to one by zeroing some saturation constants or removing unnecessary terms, upon the biological premises. A detailed description of this operation is presented in Table 1.

Taking all the additional assumptions collected in Table 2 into account, the model equations were simplified to:

$$\frac{dc_{LAC}}{dt} = -\frac{1}{Y_{X/LAC}} \cdot \mu_{\max} \cdot \frac{c_{GLC}}{c_{GLC}+K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N+K_N^{X} \cdot c_X} \cdot \frac{K_{I,GLC,1}}{K_{I,GLC,1}+c_{GLC}} \cdot c_X - \\ -\frac{1}{Y_{LOV/LAC}} \cdot q_{\max}^{LOV} \cdot \frac{c_{LAC}}{c_{LAC}+K_{LAC}^{LOV} \cdot c_X} \cdot \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV}+c_N} \cdot \frac{K_{I,GLC,2}}{K_{I,GLC,2}+c_{GLC}} \cdot c_X \tag{6}$$

Table 1. Simplification of the model: omission of selected limitation terms

Parameter zeroed	Balance of …	Biological reason
K_{LAC}^{X} K_{GLY}^{X}	biomass	Both substrates are in excess, although glycerol is used up first. It is obvious that biomass growth ceased due to the lack of nitrogen.
K_{LAC}^{X}	lovastatin (growth associated product formation term)	In the trophophase lactose is in excess and hardly utilised, so it cannot limit product formation.
K_{LOV}^{GLC}	lovastatin (non-growth associated product formation term)	Glycerol does not take part in this phase as it is depleted; lactose is the main substrate for product formation in this phase.
K_{GLY}^{X}	nitrogen	In the early trophophase when nitrogen is utilised, glycerol is in excess, later only lactose may influence its uptake.
K_{LAC}^{X}	glycerol (substrate utilisation for growth)	When glycerol is utilised, lactose is in excess.
K_{LAC}^{X}	lactose (substrate utilisation for growth)	Biomass growth rate is practically independent of lactose concentration as it is in excess. Growth can be only limited by glycerol.
K_{GLY}^{LOV}	lactose (substrate utilisation for product formation)	Glycerol is no longer present in the system, when lactose is utilised for lovastatin production.
K_{LAC}^{LOV}	glycerol (substrate utilisation for product formation)	Lactose cannot limit product formation, when lovastatin is formed on glycerol as it is then in excess.

Table 2. Parameters of the model estimated on the basis of runs *AB1*, *AB2* and *AB3*

Parameter	Value
Maximum specific biomass growth rate, μ_{max}, h^{-1}	0.12
Maximum specific formation rate of lovastatin, q_{max}^{LOV}, g LOV g X^{-1} h^{-1} *	0.00178 0.00140 0.00090
Biomass to nitrogen yield coefficient, $Y_{X/N}$, g X g N^{-1}	18.0
Biomass to lactose yield coefficient, $Y_{X/LAC}$, g X g LAC^{-1}	0.55
Biomass to glycerol yield coefficient, $Y_{X/GLC}$, g X g GLC^{-1}	0.55
Lovastatin to lactose yield coefficient, $Y_{LOV/LAC}$, g LOV g LAC^{-1}	0.0065
Lovastatin to glycerol yield coefficient, $Y_{LOV/GLC}$, g LOV g GLC^{-1}	0.0050
Lovastatin to biomass yield coefficient, $Y_{LOV/X}$, g LOV g X^{-1} *	0.0052 0.0025 0.0025
Contois type saturation of lactose towards biomass, K_{LAC}^{X}, g LAC g X^{-1}	1.63
Contois type saturation of glycerol towards biomass, K_{GLC}^{X}, g GLC g X^{-1}	0.01
Contois type saturation of nitrogen towards biomass, K_{N}^{X}, g N g X^{-1}	0.07
Contois type saturation of lactose towards lovastatin, K_{LAC}^{LOV}, g LOV g X^{-1}	12.0
Contois type saturation of glycerol towards lovastatin, K_{GLC}^{LOV}, g LOV g X^{-1}	12.0
Glycerol inhibition constant, $K_{I,GLC,1}$, g GLC l^{-1}	0.000018
Glycerol inhibition constant, $K_{I,GLC,2}$, g GLC l^{-1}	0.00010
Nitrogen inhibition constant towards lovastatin, $K_{I,N}^{LOV}$, g N l^{-1}	0.29

* These two parameters had to be tuned dependent on the ratio of glycerol and lactose used initially as carbon sources in the runs *AB1*, *AB2* and *AB3*, respectively

$$\frac{dc_{GLC}}{dt} = -\frac{1}{Y_{X/GLC}} \cdot \mu_{\max} \cdot \frac{c_{GLC}}{c_{GLC} + K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N + K_N^X \cdot c_X} \cdot c_X - \frac{1}{Y_{LOV/GLC}} \cdot q_{\max}^{LOV} \cdot \frac{c_{GLC}}{c_{GLC} + K_{GLC}^{LOV} \cdot c_X} + \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV} + c_N} \cdot c_X \quad (7)$$

$$\frac{dc_N}{dt} = -\frac{1}{Y_{X/N}} \cdot \mu_{\max} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC}^{X} \cdot c_X} \cdot \frac{c_N}{c_N + K_N^X \cdot c_X} \cdot c_X \quad (8)$$

$$\frac{dc_{LOV}}{dt} = Y_{LOV/X} \cdot \mu_{\max} \cdot \frac{c_{GLC}}{c_{GLC} + K_{GLY}^{X} \cdot c_X} \cdot \frac{c_N}{c_N + K_N^X \cdot c_X} \cdot c_X + q_{\max}^{LOV} \cdot \frac{c_{LAC}}{c_{LAC} + K_{LAC}^{LOV} \cdot c_X} \cdot \frac{K_{I,N}^{LOV}}{K_{I,N}^{LOV} + c_N} \cdot c_X \quad (9)$$

$$\frac{dc_X}{dt} = \mu_{\max} \cdot \frac{c_N}{c_N + K_N^X \cdot c_X} \cdot c_X \quad (10)$$

For the sake of model simplification it was also assumed that K_{GLC}^{LOV} is equal to K_{LAC}^{LOV}.

4.3. Determination of the model parameters and simulations

The model parameters were determined on the basis of the experimental data from the runs *AB1*, *AB2* and *AB3* conducted in the shake flask (Table 2) using the numerical methods described in *Materials and methods* section. In Fig. 4 simulated curves and experimental points are shown.

The quality of the fit seemed to be satisfactory and an in-depth discussion is going to be performed later.

4.4. Verification of the model

For the purpose of model verification the independent runs *BB1*, *BB2* and *BB3* performed in a 5.3 litre batch bioreactor were used. In the simulation two parameters of the model were tuned, i.e. $q_{max}^{LOV} = 0.00085$ g LOV g X^{-1} h^{-1} and $Y_{LOV/X}$=0.0009 g LOV g X^{-1}.The simulation curves and experimental data are shown in Fig. 5. Despite the fact that the conditions in the bioreactor were different than those in the shake flasks and additionally *pH* control and pO_2 control were used the fit of the model to the experimental data was is fairly good, excluding the overestimation of lovastatin production in run *BB2*. The need of decreasing the values of the two parameters and a worse fit will be discussed in the next section.

4.5. Discussion

The discussion on the aforementioned results is going to be divided into two parts. First, some comments will be presented with regard to the parameters of the model. Second, the issue of biomass growth kinetics will be discussed in a wider apprehension.

To start with, it must be mentioned that it was impossible to describe the experimental data with the same set of parameters, even for the shake flask culture. Two parameters connected with lovastatin formation, namely maximum specific formation rate of lovastatin q_{max}^{LOV} and yield coefficient lovastatin over biomass $Y_{LOV/X}$ had to be varied dependent on the run. It was found that the more glycerol was used in the initial phase of cultivation, the lower was non-growth associated maximum specific formation rate of lovastatin. It may indicate a higher activity of the fungus in the trophophase and subsequently worse lactose utilisation in the idiophase. It is clearly seen in Fig. 2 that the shapes of lovastatin curve within the first 72 hours slightly differed in these three runs. As lovastatin formation is strictly connected with substrate utilisation this behaviour of the model seems to be justified. Glycerol somewhat dominated over lactose, if present at higher concentrations at the beginning of the run. Another situation was observed with $Y_{LOV/X}$. The more glycerol was used, the lower was this coefficient. Although more glycerol was present at the initial stages of growth, lovastatin formation was not more

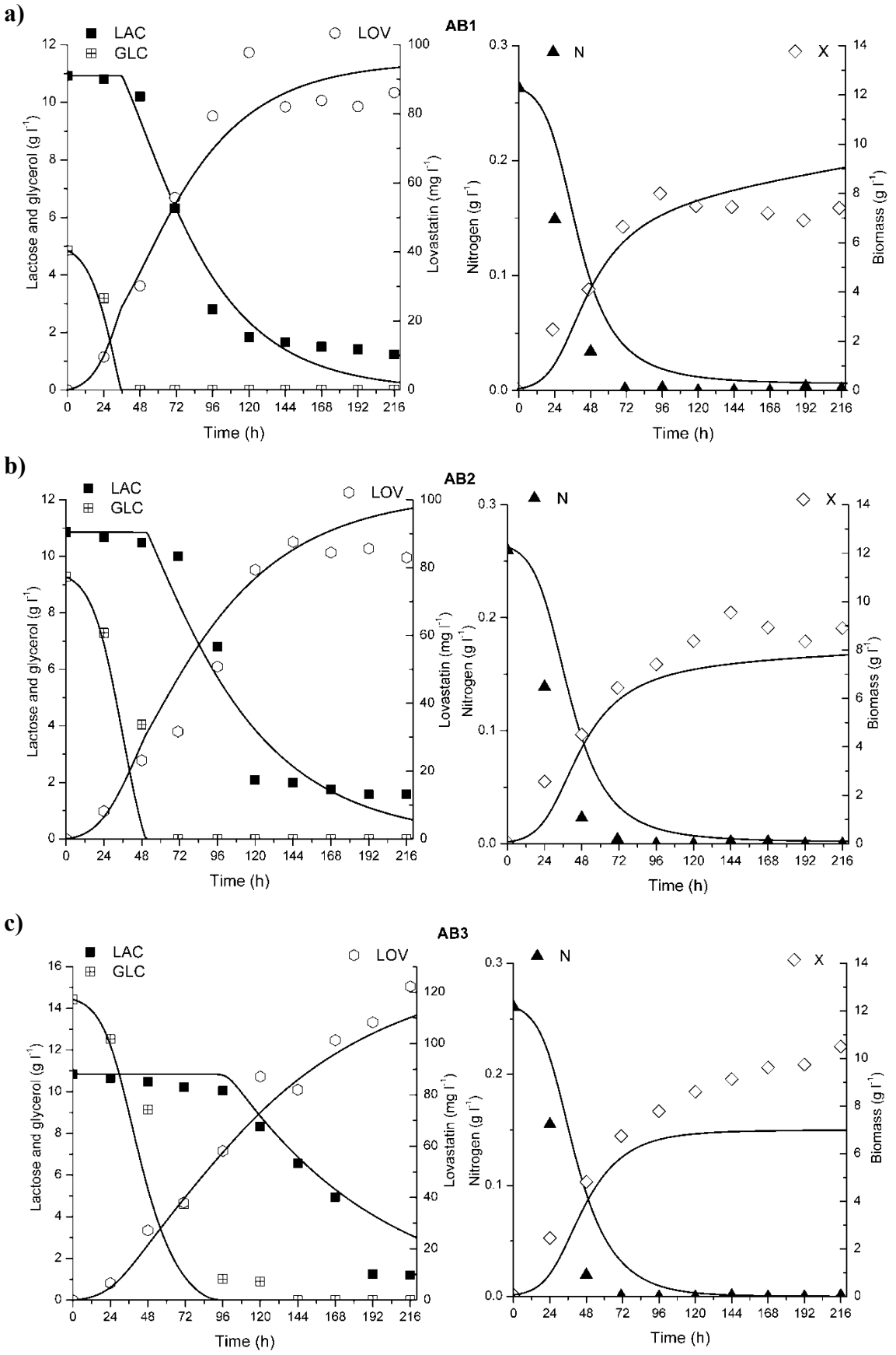

Fig. 4. Comparison of simulated curves and experimental points for the runs *AB1* (a), *AB2* (b) and *AB3* (c)

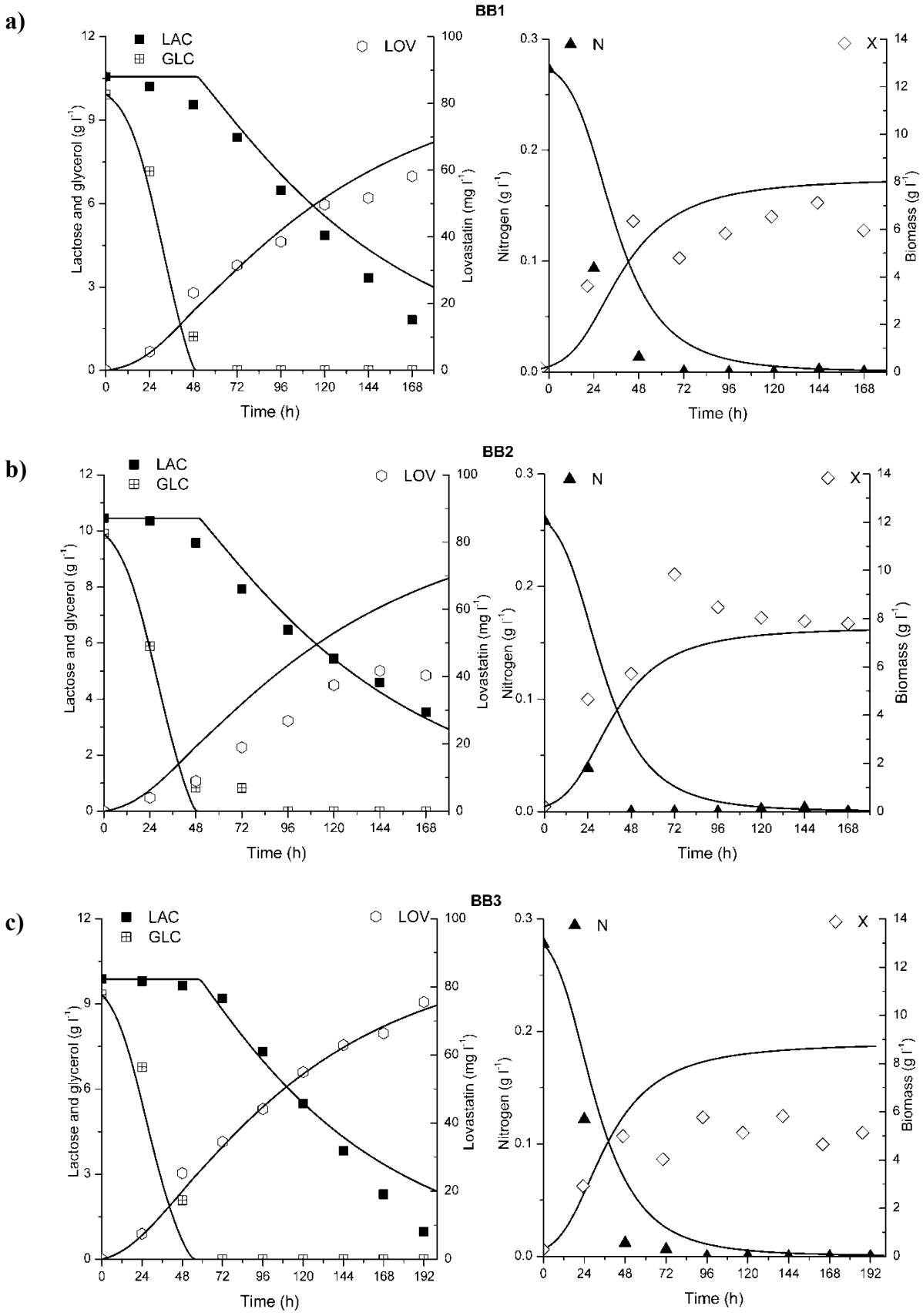

Fig. 5. Comparison of simulated curves and experimental points for the runs *BB1* (a), *BB2* (b) and *AB3* (c). In all these runs pO_2 was controlled at 20% saturation and *pH* was set from 24 hour of the run at 6.95, 7.0 and 7.1 respectively; two parameters q_{max}^{LOV}=0.00085 g LOV g X^{-1} h^{-1} and $Y_{LOV/X}$=0.0009 g LOV g X^{-1} were tuned compared to the simulation for shake flask culture

efficient. It somewhat confirms the well known fact that glycerol is a more suitable substrate for lovastatin production if it is added to a culture in the idiophase (Manzoni et al., 1998). It was also confirmed by Pecyna and Bizukojć (2011) in fed-batch experiments. These two parameters q_{max}^{LOV} and $Y_{LOV/X}$ were also tuned in the verification of the model using three independent bioreactor runs. Their values are lower than those in the shake flask culture. What is more, in run *BB2* less lovastatin was formed than the simulation predicted. This phenomenon was somewhat expected. Recently, Pawlak and Bizukojć (2012) presented a detailed study of increasing bioreactor scale for lovastatin production, showing that usually in bioreactors lovastatin titres are lower that those in shake flasks and additional measures, especially *pH* control with bicarbonate solution, must be undertaken to get closer to the titres easily obtained in shake flasks. The reason for this phenomenon must be sought on the intracellular level (not fully described yet). That is why this model, as unstructured one, could not predict that and two parameters connected with lovastatin production had to be decreased.

With regard to biomass, both substrates seem to be equally efficient as both yield coefficients for biomass formation on these substrates were assumed to be equal. Nevertheless, it must be remembered that it was glycerol, which contributed more to biomass growth and it is clear that if these substrates were used independently, these coefficients would be different, which can be concluded from the previous work (Bizukojć and Pecyna, 2011). Biomass to nitrogen coefficient was similar to the one previously found by Bizukojć and Ledakowicz, 2007.

It is not surprising that lovastatin yield on lactose $Y_{LOV/LAC}$ was higher than that on glycerol $Y_{LOV/GLC}$. It remains in agreement with the results of the previous experiments performed by Casas López et al. (2003) and Bizukojć and Pecyna (2011). Lactose is a better substrate if used separately. Nevertheless, the enhancing effect of these two substrates was observed if both were used in the medium.

Low levels of all saturation constants K_{LAC}^{X}, K_{GLC}^{X} and K_{N}^{X} with regard to biomass growth was a typical feature of this parameter for such easily utilisable substrates as lactose or glycerol. It is enough to compare even lower Monod saturation constants cited by Nielsen et al. (2003). On the other hand, the saturation constants for lactose and glycerol in lovastatin formation K_{LAC}^{LOV} and K_{GLC}^{LOV} were quite high. It again confirmed how important high substrate flux for *A. terreus* to produce lovastatin is. In the light of this finding it is understandable, why other authors use even more concentrated media with lactose levels reaching even over 100 g l^{-1} (Casas López et al, 2005; Lai et al., 2005). They only have to supply more oxygen to oxidise this substrate, which is possible only in effectively aerated bioreactors (Casas López et al, 2005). Extremely low levels of glycerol inhibition or, to use a more suitable word, repression constant confirmed the run of the experiment. Lactose was hardly consumed unless all glycerol had been depleted. Nitrogen inhibition constant had to be used to satisfy the inhibitive effect of organic nitrogen on lovastatin formation (Bizukojć and Ledakowicz, 2007)

The fit of biomass curve was the worst of all. Its reason is the aforementioned untypical biomass growth due to mass transfer resistance. Using such modelling approach as shown in this work, it was impossible to get better results. Looking closer into these mass transfer issues, one may notice that the main problem is that *A. terreus* grew in the form of macroscopic pellets of diameter of 1 to 4 mm. That is why substrate, e.g. oxygen, gradient was always observed in these pellets. In bigger pellets the zone without oxygen could be found in their centres. It is shown in Fig. 6 upon our own experimental data.

Of course it cannot be excluded that this gradient also occurred for other substrates such as lactose, glycerol or any other nutrient. Here, the technical limitations prevented us from measuring them. All in all, mass transfer resistance in the pellets led to the situation that a truly exponential growth was observed only within the first 12 hours of the runs. Later in the trophophase due to these limitations in the transfer of substrates into the centres of pellets biomass growth became linear (Fig. 3). It must be also mentioned that the literature provides hardly any model that takes such effects connected with mass transfer into account, mainly due to the difficulties to reliably determine mass transfer coefficient in the pellet. And finally in the light of the aforementioned discussion it is not astonishing that smaller

pellets with fewer limitations of mass transfer are more favourable for lovastatin production (Bizukojć and Ledakowicz, 2010).

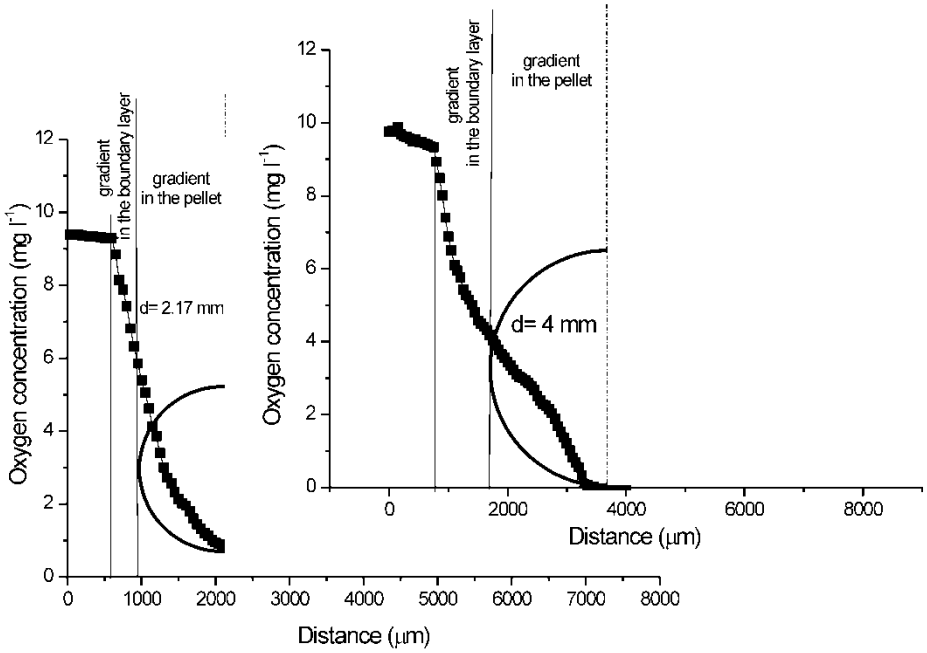

Fig. 6. Oxygen profile in the pellets of various diameters: 2.17 and 4 mm

5. CONCLUSIONS

Upon the findings presented in this paper the following conclusions can be drawn:

- The proposed model, despite its simplicity (unstructured approach) described the experimental data with the satisfactory accuracy with regard to substrate utilisation and product formation. Furthermore, its verification for the bioreactor batch system proves to be good.
- All the assumptions of the model are in agreement with the contemporary knowledge on lovastatin biosynthesis by *A. terreus* and the parameters determined have their biological sense. They enhance the value of this model.
- This model can be useful to design valid feeding functions in fed-batch systems fed with carbon substrates.
- The limitations of the unstructured modelling approach in the description of such pelleted fungal systems as *A. terreus*, especially with regard to biomass growth kinetics, are clearly indicated. Consequently, this model should be also treated as a premise to undertake further actions leading to take substrate diffusion in pellets into account.

The authors wish to acknowledge National Science Centre (Republic of Poland) for the partial financial support of this work, project no. N N209 765240.

SYMBOLS

c_{GLC} glycerol concentration, g GLC l^{-1}
c_{LAC} lactose concentration, g LAC l^{-1}

c_{LOV} lovastatin concentration, mg LOV l^{-1}
c_N nitrogen concentration, g N l^{-1}
c_X biomass concentration, g X l^{-1}
K_{GLC}^{LOV} Contois type saturation of glycerol towards lovastatin, g LOV g X^{-1}
K_{GLC}^{X} Contois type saturation of glycerol towards biomass, g GLC g X^{-1}
$K_{I,GLC,1}$ glycerol inhibition constant, g GLC l^{-1}
$K_{I,GLC,2}$ glycerol inhibition constant, g GLC l^{-1}
$K_{I,N}^{LOV}$ lovastatin inhibition constant, g LOV l^{-1}
K_{LAC}^{LOV} Contois type saturation of lactose towards lovastatin, g LOV g X^{-1}
K_{LAC}^{X} Contois type saturation of lactose towards biomass, g LAC g X^{-1}
K_N^X Contois type saturation of nitrogen towards biomass, g N g X^{-1}
q_{max}^{LOV} maximum specific formation rate of lovastatin, g LOV g X^{-1} h^{-1}
r_{GLC} volumetric glycerol uptake rate, g GLC l^{-1} h^{-1}
r_{LAC} volumetric lactose uptake rate, g LAC l^{-1} h^{-1}
r_{LOV} volumetric lovastatin formation arte, mg LOV l^{-1} h^{-1}
r_N volumetric glycerol uptake rate, g N l^{-1} h^{-1}
r_X volumetric biomass growth rate, g X l^{-1} h^{-1}
$Y_{LOV/GLC}$ lovastatin to glycerol yield coefficient, g LOV g GLC^{-1}
$Y_{LOV/LAC}$ lovastatin to lactose yield coefficient, g LOV g LAC^{-1}
$Y_{LOV/X}$ lovastatin to biomass yield coefficient, g LOV g X^{-1}
$Y_{X/GLC}$ biomass to glycerol yield coefficient, g X g GLC^{-1}
$Y_{X/LAC}$ biomass to lactose yield coefficient, g X g LAC^{-1}
$Y_{X/N}$ biomass to nitrogen yield coefficient, g X g N^{-1}

Greek symbols

μ_{max} maximum specific biomass growth rate, h^{-1}

Subscripts

GLC glycerol
LAC lactose
LOV lovastatin
N nitrogen
X biomass

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Received 16 May 2012
Received in revised form 13 November 2012
Accepted 21 November 2012