CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438032

Integrated Microbial Process for Bioconversion of Crude Glycerol from Biodiesel into Biosurfactants and PHAs

Marco S. Nicolò[a], Domenico Franco[a], Viviana Camarda[a], Rosa Gullace[a], Maria G. Rizzo[a], Manuela Fragalà[b], Grazia Licciardello[b], Antonino F. Catara[b], Salvatore P.P. Guglielmino[b*]

[a]Dipartimento Scienze Biologiche ed Ambientali, Università di Messina
[b]Parco Scientifico e Tecnologico della Sicilia,Catania
sguglielm@unime.it

Biodiesel production from oils and fats is continuously growing, raising the problem on how to dispose crude glycerol, the main byproduct, in an economic and ecofriendly way. On the parallel, the demand of green chemicals, such as polyhydroxyalkanoates (PHAs) and biosurfactants (BSs), is increasing, becoming a valuable economic perspective for new industrial processes.
Some bacteria are able to synthesize PHAs and BSs by different carbon sources, therefore crude glycerol may be a suitable source for high-value added green chemicals.
In this work, crude glycerol from *Brassica carinata* oil was converted into PHAs and BSs by *Pseudomonas mediterranea* 9.1. Addition of either meat or yeast extracts improved glycerol fermentation, minimizing the prolonged lag phase observed in mineral medium. The effects of nutritional requirements, pH, temperature and fermentation time on the PHA and BSs yields were evaluated by response surface methodology (RSM). A Box-Behnken experimental design was adopted to derive a statistical model for variables optimization. In order to establish an accurate control of PHAs and BSs yields and fermentation time, the data from RSM were applied to a mathematical mechanistic model, in order to simulate three distinct fermentative processes, which were verified experimentally, to obtain i) maximum production of PHA, ii) maximum production of BSs and iii) co-production of both. Results showed that *P. mediterranea* used crude glycerol as carbon source, at a concentration of 2.5%, and was able to convert it into PHA and BSs. The data from mathematical mechanistic model, experimentally confirmed by batch fermentations, indicated that maximum production of PHA (1.63 g/L) required 48 h with 2.5 mM $(NH_4)_2HPO_4$, 0.1 % meat or yeast extracts, pH=6.9 and T=27°C, whereas maximum production of BSs (0.8 g/L) required 96 h with 8 mM $(NH_4)_2HPO_4$, 0.1% meat or yeast extracts, pH=7.1 and T=33°C.
In the co-production process, satisfactory yields of both PHA (1.1 g/L) and BSs (0.72 g/L) were simultaneously produced within 72 hours. The co-production strategy in a single integrated fermentative process looks as promising for industrial production of green chemicals at competitive costs.

1. Introduction

Bacterial polyhydroxyalkanoate biopolymers (PHAs) are renewable and ecofriendly biomaterials and may replace plastics in several fields, as packaging, food services, bio-medical, and agriculture industries.
Biosurfactants (BSs) are microbial compounds, mainly consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins. The chemical composition depends on the producer microrganism, the starting substrate and the process conditions and corresponds to a wide diversity of applications.
A market report (www.marketsandmarkets.com) evaluated that PHA market consumption will grow from an estimated 10,000 tons in 2013 to 34,000 tons by 2018, whereas the global biosurfactants market was worth USD 1,735.5 million in 2011 and will reach USD 2,210.5 million in 2018 (www.transparencymarketresearch.com). PHA market, although in an embryonic stage, is characterized by rapid technological development and increasing investment in research.

Please cite this article as: Nicolò M.S., Franco D., Camarda V., Gullace R., Rizzo M.G., Fragala M., Licciardello G., Catara A., Guglielmino S.P.P., 2014, Integrated microbial process for bioconversion of crude glycerol from biodiesel into biosurfactants and phas, Chemical Engineering Transactions, 38, 187-192 DOI: 10.3303/CET1438032

On the parallel, also the market of biofuels, as biodiesel, is constantly growing and their production is estimated to reach 1,900 million barrels in 2020.
Biodiesel is produced by transesterification of vegetable oils or animal fats. Crude glycerol is the main by-product and represents approximately 16 to 18% in weight of the input of the oil/fat.
The microbial conversions of crude glycerol into PHA and BSs are promising. So, new strategies are required to improve the industrial production of such compounds to allow their widespread diffusion in many fields and applications.
For this purpose, we developed a fermentation process for simultaneous production of both PHA and BSs by crude glycerol using a strain of *Pseudomonas mediterranea*, already tested for its ability to synthesize PHA from crude glycerol (Palmeri et al., 2012).
Glycerol metabolism by several pseudomonads implies a quite prolonged lag-phase, due to catabolite repression control (Nikel et al., 2013), that can be abolished by a so-called “good” carbon source.
It is known that phytopathogen pseudomonads use aminoacids from root exudates as carbon and nitrogen sources (Sonawane et al., 2003). Amino acids are a good source of carbon, able to abolish the catabolite repression control (Moreno et al., 2009). Therefore, we added a source of aminoacid mixtures, i. e. meat or yeast extract, in the medium formulation and a significant reduction of lag-phase was observed when glycerol was used as the sole carbon source.
In order to optimize a process for PHA and BSs coproduction, a response surface methodology (RSM) was used to select the best values of each chemo-physical variable. RSM is a group of statistical and mathematical procedures, aiming to study the relationships between one or more responses (e. g. biomass, PHA and BSs productions) and a defined number of independent variables (i. e., nutritional and chemo-physical parameters). Such methodology generates a mathematical model that accurately describes the overall process (Senanayake and Shahidi, 2002).
Thereafter, the statistically consistent data obtained from RSM analysis were subsequently analyzed by a mathematical mechanistic model (Vázquez and Murado, 2008), with some modifications to correlate carbon and nitrogen consumption to biomass, PHA and BSs productions under different chemo-physical conditions. Such modified model was then used to generate three different *in silico* simulations to get *i*) maximum production of PHA, *ii*) maximum production of BSs and *iii*) co-production of both.

2. Materials and methods

2.1 Experimental methods

An ubiquitous strain of *P. mediterranea*, namely 9.1, was cultivated in LB broth, at 30°C under shaking for 15-18 h. All the experiments were led in mineral medium containing the following: 5.8 g/L K_2HPO_4; 3.7 g/L KH_2PO_4; 10 mL/L 0.1 M $MgSO_4$; 1 mL/L of a microelement (MT) solution. MT solution contained the following: 2.78 g/L $FeSO_4 \cdot 7H_2O$; 1.98 g/L $MnCl_2 \cdot 4H_2O$; 2.81 g/L $CoSO_4 \cdot 7H_2O$; 1.67 g/L $CaCl_2 \cdot 2H_2O$; 0.17 g/L $CuCl_2 \cdot 2H_2O$; 0.29 g/L $ZnSO_4 \cdot 7H_2O$.
At first, crude glycerol (1% w/v) and its main components, namely pure glycerol (1% w/v), erucic, miristic, stearic, linoleic and palmitic acids (10 mM each) were tested as carbon sources for PHA and BSs production. 1 liter-cultures in mineral medium, supplemented with 1.1 g/L $(NH_4)_2HPO_4$ (E* medium) were incubated at 30°C for 72 h under shaking.
In order to reduce the lag-phase, typical of glycerol fermentation, meat or yeast extracts were added to the mineral medium as source of amino acids.
PHA and BSs were evaluated by Nile Red staining (Degelau et al., 1995; Spiekermann et al., 1999) and Emulsion Index (E_{24}) (Cooper and Goldenberg, 1987), respectively, and results were expressed as percentages.

2.2 Value range of chemophysical parameters

Before RSM analysis, a Box-Behnken design (Box and Behnken, 1960) was developed for the following value ranges, using Minitab 16 software: glycerol (0.25-4.5% w/v), $(NH_4)_2HPO_4$ or NH_4NO_3 (1.25-80 mM), yeast or meat extract (0.01-0.1% w/v), pH (6-8), temperature (25-37°C). Each range was determined by varying one factor at a time while keeping the others constant. The incidence of nutritional factors and chemo-physical parameters, each considered as an independent variable, was evaluated by Box-Behnken design with three uncoded-levels and analyzed by response surface regression in Minitab software.
Biomass, PHA and BSs yields were considered as dependent variables or “responses”.
Experimental design was evaluated as optical density at 540 nm (OD_{540}) by automated turbidometer (Labsystems Bioscreen C). Each sample, in ten replicates, was incubated under shaking, for a period of 120 h.

The unstructured mechanistic model proposed by Vázquez and Murado (2008) was adapted to the most incident factors. It describes microbial kinetics and includes the most fundamental observations relating to microbial growth processes: (a) the biomass concentration and the rate of cell mass production are proportional; (b) the cells need substrate and can synthesize metabolic products even when growth has finished; (c) the evolution of the biomass throughout the culture time (growth rate) presents an asymptote as upper limit (saturation level) different for each substrate or level of substrate used.
Briefly, the following logistic equation, Eq(1), which expresses the biomass as a function of the time (Vázquez and Murado, 2008), was utilized for the study of growth kinetics, while the Luedeking and Piret equation, Eq(2), was utilized to evaluate the rate of productions of PHA and BSs.

$$X = \frac{K}{1 + exp\ [2 + \mu \cdot (\lambda_X - t)]} \quad (1)$$

where X is biomass (g/L), K the maximun yield of biomass (g/L), μ the specific growth rate (1/generation time, h^{-1}) and λ_X is the lag-phase (h);

$$r_P = \frac{dP}{dt} = \alpha_P \cdot \frac{dX}{dt} + \beta_P \cdot X \quad (2)$$

where r_P is the production rate, expressed as temporal variation of product arbitrary units (PU) per ml (PU ml^{-1} h^{-1}), α_P is the growth-associated constant for product production (PU mg^{-1}), β_P non growth-associated constant for product production (PU mg^{-1} h^{-1}), X biomass (g/L).
Since nitrogen and carbon source are related to biomass, PHA and BSs productions and to cellular maintenance, their uptakes were modelled by the following equation, Eq(3):

$$S = S_0 + \frac{X_0}{Y_{X/S}} - \frac{1}{Y_{X/S}} \cdot \frac{K}{1 + \left(\frac{K}{X_0} - 1\right) \cdot e^{-\mu_{mX} \cdot t}} - \frac{m_S \cdot K}{\mu_{mX}} \cdot ln\left[\frac{X_0 \cdot (e^{\mu_{mX} \cdot t} - 1) + K}{K}\right] \quad (3)$$

where S_0 is the initial substrate concentration (g/L), X_0 is the initial biomass concentration (g/L), $Y_{X/S}$ is the conversion rate of substrate into biomass and mS is maintenance coefficient (g glycerol g^{-1} biomass h^{-1}).
Fitting procedures and parametric estimations calculated from the results were carried out by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method.
Data from the model were then verified in a Biostat MD 3-liters bioreactor, with single batch fermentative processes. At the end of fermentation period, PHA extraction and BSs recovery were performed for quantitative analysis.
PHA was extracted from cells harvested by centrifugation at 8,000 *xg* for 10 minutes, resuspended in a minimal volume of sterile milliQ water, then lyophilized. Dried biomass was dissolved in acetone (Jiang et al, 2006), and refluxed in Soxhlet extractor at boiling point (56° C) for 6 h. The lipidic extract was then dried under vacuum by rotavapor until the weight was kept constant.
BSs were recovered by an adsorption-desorption technique, using wood-based activated carbons (WACs) (Dubey et al, 2005), modified with a thermal pretreatment of WACs. Thermally pretreated WACs (tp-WACs) at 5% w/v concentration were added to flasks containing 100 ml of cell-free supernatant. Flasks were shaken at 150 rpm and 40° C for 90 minutes. After the contact time, the tp-WACs were separated through Whatman filter paper No. 42, washed with distilled water to remove unadsorbed BSs and suspended in stoppered flasks containing 20 ml of acetone. The flasks were agitated at 100 rpm for 6 h at 30° C. The acetone extract was separated from the adsorbent, which was washed at least three times with fresh acetone. All the acetone extracts from one sample were unified and acetone was removed under vacuum.

3. Results and discussion

3.1 Nutrients optimization

Preliminarily, the ability of *P. mediterranea* to degrade the main components of crude glycerol, e.g. pure glycerol and myristic, erucic, stearic, linoleic and palmitic acids, and convert them into PHA was evaluated at 72 h (Table 1).

Table 1: Biomass and PHA yields from different carbon sources in E medium after 72 h*

Substrate	Biomass yield (g/L)	PHA yield (g/L)
Pure glycerol (1% w/v)	1.56	0.32
Miristic acid (10mM)	0.82	0.09
Erucic acid (10mM)	2.05	0.13
Stearic acid (10mM)	1.41	0.13
Linoleic acid (10mM)	0.98	>0.001
Palmitic acid (10mM)	0.93	0.09
Crude glycerol (1% w/v)	1.81	0.57

Although *P.mediterranea* was able to grow and produce PHA with all the tested carbon sources, a prolonged lag-phase (5-12h) was observed.
It is known that aminoacids are a source of both nitrogen and carbon for several pseudomonads (Moreno et al., 2009; Sonawane et al., 2003). Therefore, we assumed that they could be used as a "good" carbon source by *P. mediterranea* to reduce the lag-phase, favoring the uptake of glycerol. At this purpose, rich sources of aminoacids, as yeast or meat extract, were added to the medium, using glycerol as primary carbon source. Indeed, the experimental data showed that lag-phase was abolished when glycerol fermentation was carried out adding 0.1% meat or yeast extracts.
RSM analysis for biomass, PHA and BSs, performed in separate batch fermentations, showed that nitrogen and carbon concentrations were the mostly incident variables.
In particular, carbon concentration below 1.5% w/v negatively influenced biomass production, whereas, in the range 1.5-4.5% w/v, no further increase in biomass was observed. PHA yield was positively influenced in the range 1.5-2.5% w/v and no further increase was observed with higher concentrations, at least until the concentrations of nitrogen were below 20 mM. Such results are in accordance with the general knowledge that PHA production is strictly related to an excess of carbon source (high C:N ratio).
About nitrogen inorganic source, NH_4NO_3 and $(NH_4)_2HPO_4$ showed similar efficiency in specific maximum growth rate (μ), however, the latter was preferred, because the biomass yield was significantly higher (25%). On the other hand, nitrogen concentrations below 5mM were related to a maximum PHA production, whereas nitrogen concentration in the range 10-160 mM positively influenced the yields of both biomass and BSs. In fact, it is known that BSs production is under control of quorum sensing, which is triggered in high-density populations.

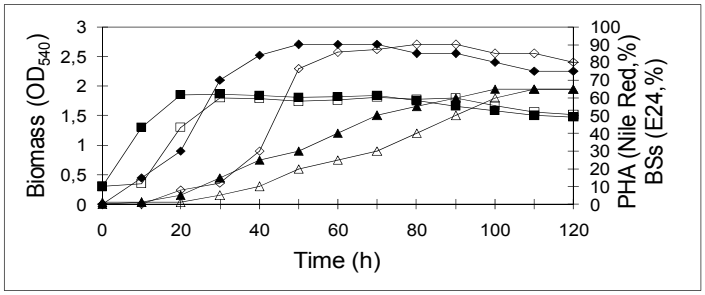

Figure 1: Production kinetics of biomass (■,□), PHA (♦,◊) and BSs (▲,Δ) in E-medium with (full symbols) and without (open symbols) 0.1% meat or yeast extract.

Meat or yeast extract had no effect in reducing the lag-phase at concentration of 0.01%, whereas at 0,05%-0.1% this was abolished, allowing the maximal growth of biomass within 18 h, whereas cultures in standard E* medium required 29 h to achieve the maximum of biomass. As consequence, production of both PHA and BSs was brought forward 11 hours (Figure 1). Moreover, when yeast or meat extracts were added in presence of 5 mM nitrogen at concentrations above 0.1% w/v, PHA synthesis was proportionally delayed. In fact, the nitrogen contribute of the extracts was conveyed towards biomass production, as by experimental proofs.
The obtained data showed that pH and temperature mutually influenced PHA and BSs production in a narrow range of variation. The condition of pH= 6.9 and temperature= 27°C positively influenced PHA production, whereas the condition of pH= 7.1 and temperature= 33°C increased BSs synthesis.
The experimental data were used to set up a mathematical mechanistic model, which showed the consumption of nitrogen and carbon sources, related to other variables, as a function of the growth trend

and PHA and biosurfactants production. In doing so, it was possible to simulate *in silico* the impact of different values of the considered variables on the whole process. As an example, a simulation, with the following parameters: 1.5% glycerol, 8 mM ammonium (without meat or yeast extracts), T= 30°C and pH 7 is plotted in Figure 2.

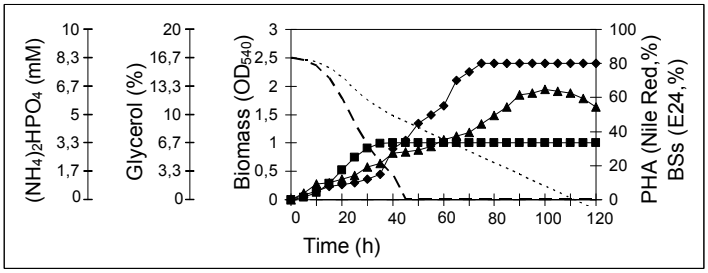

Figure 2: Simulation of production kinetics of biomass (■), PHA (♦), BSs (▲), and nitrogen (discontinuous line) and glycerol uptake (dotted line) in mineral medium.

The *in silico* data were confirmed in experimental tests, allowing fine adjustment of the entire process. Consequently, three different parameterizations of temperature, pH and nitrogen concentration were fitted to obtain: i) maximum production of PHA (Figure 3, left), ii) maximum production of BSs (Figure 3, middle) or iii) co-production of both in a unique integrated process (Figure 3, right).

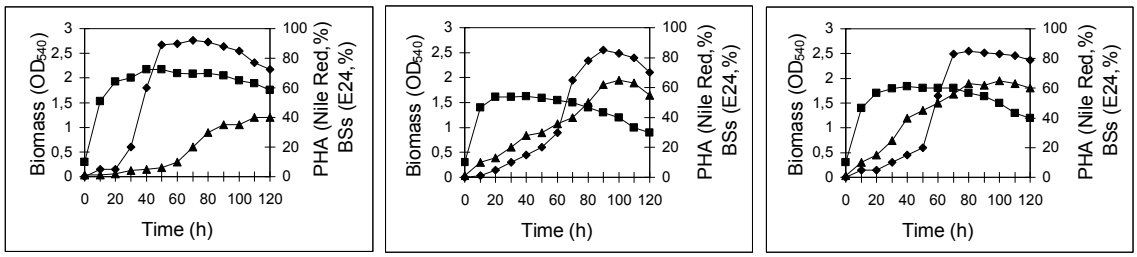

Figure 3: Production kinetics of biomass (■), PHA (♦) and BSs (▲) production under different parameterizations of temperature, pH and nitrogen concentration (experimental data, see text for details).

By varying the values of temperature, pH and nitrogen concentration, in batch fermentation, the highest PHA yield on CDW (60%, 1.63 g/L) was obtained after 48 h using 2.5% crude glycerol, 2.5mM $(NH_4)_2HPO_4$, 0.1 % meat or yeast extracts, pH=6.9 and T=27°C ; the highest biosurfactants yield (0.8 g/L) after 96 h, using 2.5% crude glycerol, 5mM $(NH_4)_2HPO_4$, 0.1% meat or yeast extracts, pH=7.1 and T=33°C.
The integrated process for co-production of both PHA and BSs was performed in a 3-L bioreactor, with the following parameters: crude glycerol 2,5%, 4 mM $(NH_4)_2HPO_4$, 0.1 % meat or yeast extracts, pH 7 +/- 0.1, temperature 31° C, pO_2 20%, airflow 0.3 l min-1, stirring at 200 rpm (in cascade with pO_2), for 72 h.
Finally, under such conditions, yields were: 2.2 g/L for biomass, 1.1 g/L for PHA (50% on CDW) and 0.72 g/L for BSs.

4. Conclusion

The interest in potential applications for PHA and BSs by numerous industries has recently increased, due to the characteristics of sustainability and respect for the environment.
Coproduction of PHA and rhamnolipids from decanoate by *Pseudomonas aeruginosa* has been described in a two-stage process, in which PHA accumulation followed rhamnolipid production (Hori et al., 2002). As well, the same coproduction has been described, using palm oil as carbon substrate (Marsudi et al., 2008).
To lead to an improvement of the processes for synthesis and industrialization of these bioproducts, we developed a process of fermentation, by coupling the disposal of crude glycerol and the simultaneous production of both PHA and BSs, using a new bacterial strain of biotechnological interest, namely *P. mediterranea* 9.1. Exploiting the fact that pseudomonads use aminoacid as preferred carbon and nitrogen

source, we were able to reduce the fermentation time by addition of 0.1% meat or yeast extract to the mineral medium.
By informatics platform and mathematical modeling, we were able to optimize the chemo-physical variables of the process. In fact, the data obtained *in silico* were confirmed in the experimental tests, allowing fine adjustment of the entire process.
In our integrated process, we succeeded in co-production of satisfactory yields of PHA (50%) and BSs (0.72 g/L) within 72 h.
In conclusion, the strategy of coupling the disposal of crude glycerol and PHA and BSs co-production can be considered as a potential industrial process for production of high-added value green chemicals.

Acknowledgements

This work has been funded by MIUR by means of the national program PON R&C 2007-2013,project “PolyBioPlast” PON01_01377.

References

Box G., Behnken D., 1960, Some new three level designs for the study of quantitative variables, Technometrics 2, 455–475.
Cooper D.G.,Goldenberg B.G, 1987, Surface-active agents from two Bacillus species, Appl. Environ. Microbiol. 53, 224-229.
Degelau A., Scheper T., Bailey J.E., Guske C.,1995, Fluorometric measurement of poly-β-hydroxybutyrate in *Alcaligenes eutrophus* by flow cytometry and spectrofluorometry, Appl. Microbiol. Biotechnol. 42, 653–657.
Dubey K.V., Juwarkar A.A., Singh S.K., 2005, Adsorption-desorption process using wood-based activated carbon for recovery of biosurfactant from fermented distillery wastewater, Biotechnol. Prog. 21, 860-867.
Jiang X., Ramsay J.A., Ramsay B.A., 2006, Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440, J. Microb. Meth. 67, 212-219.
Hori K., Marsudi S., Unno H., 2002, Simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*, Biotechnol. Bioeng. 78, 699-707.
Marsudi S., Unno H., Hori K., 2008, Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*, Appl. Microbiol. Biotechnol. 78, 955-961.
Moreno R., Martínez-Gomariz M., Yuste L., Gil C., Rojo F., 2009, The *Pseudomonas putida* Crc global regulator controls the hierarchical assimilation of amino acids in a complete medium: Evidence from proteomic and genomic analyses, Proteomics 9, 2910–2928.
Nikel P.I., Kim J., de Lorenzo V., 2013, Metabolic and regulatory rearrangements underlying glycerol metabolism in *Pseudomonas putida* KT2440, Environ. Microbiol. 16, 239-254.
Palmeri R., Pappalardo F., Fragalà M., Tomasello M., Damigella A., Catara A.F., 2012, Polyhydroxyalkanoates (PHAs) production through conversion of glycerol by selected strains of *Pseudomonas mediterranea* and *Pseudomonas corrugata*, Chem. Eng. Trans. 27,121-126.
Senanayake S.P.J.N., Shahidi F., 2002, Lipase-catalyzed incorporation of docosahexaenoic acid (DHA) into borage oil: optimization using response surface methodology, Food Chem. 77,115–123.
Sonawane A., Klöppner U., Derst C., Röhm K.H., 2003, Utilization of acidic amino acids and their amides by pseudomonads: role of periplasmic glutaminase-asparaginase, Arch. Microbiol. 179,151–159.
Spiekermann P., Rehm B.H.A., Kalscheuer R., Baumeister D., Steinbüchel A., 1999, A sensitive, viable-colony staining method using Nile Red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds, Arch. Microbiol. 171, 73–80.
Syldatk C. and Hausmann R., 2010, Microbial biosurfactants, Eur. J. Lipid Sci. Tech., 112, 615–616.
Vázquez J.A., Murado M.A., 2008, Unstructured mathematical model for biomass, lactic acid and bacteriocin production by lactic acid bacteria in batch fermentation, J. Chem. Technol. Biotechnol. 83, 91–96.