ARTICLE DOI: 10.5504/BBEQ.2011.0019 B&E

ENHANCED PRODUCTION OF EXTRACELLULAR LIPASE BY NOVEL MUTANT STRAIN OF *ASPERGILLUS NIGER*

Lydia Toscano[1,3], Velizar Gochev[2], Gisela Montero[1] and Margarita Stoytcheva[1]
[1]Institute of Engineering, UABC, Mexicali, B.C., Mexico
[2]"Paisii Hilendarski" University of Plovdiv, Department of Biochemistry and Microbiology, Plovdiv, Bulgaria
[3]Technological Institute of Mexicali, Mexicali, B.C., Mexico
Correspondence to: Velizar Gochev
E-mail: vgochev@uni-plovdiv.bg

ABSTRACT

Twelve fungal strains belonging to 9 different species of genera Aspergillus, Pencilillium, Trichoderma and Mucor were screened for extracellular lipase production. The most active lipase producing strain A. niger was selected for strain improvement by induced mutagenesis with UV light and N-methyl-N-nitro-N-nitrosoguanidine (NMG). Chemical mutagenesis was found to be more effective in comparison to the physical one. By two stages mutagenesis with 200 μg cm^{-3} NMG for 4 h, lipase activity of parental strain was enhanced more than two times. The selected mutant A. niger $NMG_{12/4}$ was stable and maximum lipase activity of 15.5 U cm^{-3} was reached at 96 h, which corresponded to the end of the exponential growth phase. The selected mutant is prospective for the development of industrial biotechnology for production of extracellular lipase.

Biotechnol. & Biotechnol. Eq. 2011, **25**(1), 2243-2247

Keywords: *Aspergillus niger*, lipase, mutation, N-methyl-N'-nitro-N-nitroso guanidine, UV light, biodiesel

Introduction

Lipases (EC 3.1.1.3, triacylclycerol acylhydrolases) catalyze the hydrolysis of triacylglycerols to free fatty acids, diacylglycerols, monoacylgycerols and glycerol on a lipid-aqueous interface. The hydrolysis of the insoluble substrate occurs in heterogeneous conditions through nucleophilic attack on the carbonyl carbon atom from ester groups (15). Some lipases are also able to catalyze the reactions of esterification, interesterification, transesterification, acidolysis, amynolysis and may show enantioselective properties (9, 20). Lipases are widely applied for hydrolysis, synthesis, biotransformations and separations in various industries, such as food and flavor, chemical, and paper industries, wastewater treatments, as well as in scientific researches, medical diagnostic, chemical analyses and therapeutics (9, 21). Currently, biodiesel production by lipase catalyzed transesterification of triglycerides or esterification of free fatty acids is considered as a promising alternative to the chemical catalysis in biofuel synthesis. The enzymatic process allows to overcome the drawbacks of the alkali- or acid-catalyzed transesterification: product contamination, wastewater release, free fatty acids and water interferences, and difficult glycerol recovery (6, 7). However, the cost of the enzyme: approximately 1000 USD per kg of Novozym 435 lipase (6), blocks the commercialization of the lipase-catalyzed conversion. Thus, cost effective industrial production of stable lipases with enhanced activity is of primary importance.

According to the origin, lipases can be divided into three groups: plant, animal and microbial. Because of their chemical properties and stability, the most widely applied lipases are microbial (9, 21). Microbial lipases are produced by several bacteria, moulds, yeasts and actinomycetes (21, 24). Among the available lipase producing microorganisms, filamentous fungi belonging to various species of genera *Aspergillus* (1, 12), *Rhizopus* (10, 22), *Penicillium* (3, 14, 25), and *Trichoderma* (13, 18) are described as the most prospective lipase producers. The major advantages of filamentous fungi as industrial producers of lipases in comparison with the rest of microbial producers are as follows: (i) better developed secretor apparatuses and production of extracellular lipases; (ii) ability to utilize wide range of agricultural and other waste products as complex nutrition sources; (iii) fungal mycelium is easier for separation from cultural broth by vacuum filtration in comparison with bacterial and yeast biomasses, and (iv) ability to produce lipases both in submerged and solid state fermentations.

A number of publications are dedicated to the optimization of the nutritive medium composition and the cultivation conditions for lipase production by fungi (1, 9, 21, 24), but only a few studied the possibilities for enhancement of lipase production by strain improvement (2, 5, 12). Enhancement of lipase productivity of fungal strains by induced mutagenesis is a key step for the development of effective technologies for industrial scale production of lipases.

The aim of the present study is to obtain novel mutant stains with enhanced lipase activity from prospective fungal species by UV and NMG induced mutagenesis.

Materials and Methods

Microorganisms

Aspergillus awamori, *A. niger*, *A. oryzae* and four unidentified *Aspergillus* sp., *Penicillium chryzogenum*, *Trichoderma*

longibrachiatum, *T. viride*, *T. atroviride* and *Mucor* sp. were obtained from the microbial culture collection of the Department "Biochemistry and Microbiology" at "Paisii Hilendarski" University of Plovdiv (Bulgaria). The strains were maintained on Potato Dextrose Agar (PDA) and stored at 4°C.

Screening for prospective lipase producing strains

Screening for prospective lipase producing fungal species and mutant strains was carried out on tributyrin agar (BioChemica, Fluka) on the basis of the coefficient *K*, which reflects the ratio between the diameter of the clear halo around the colony (d_H, mm) and the diameter of the respective colony (d_C, mm), measured on the 7th day, at 30°C ($K=d_H/d_C$).

Conditions for UV mutagenesis

Spore suspension containing 1.5x10^5 conidia cm^{-3}, obtained from 7 days old culture of parental strain cultivated on PDA was UV treated. UV lamp GGN (Japan) 220 V, 40 W, 50 Hz was used as a source of UV light. UV mutagenesis was carried out at a distance of 20 cm from the centre of the UV lamp. The exposure time was 180 s at gentle shaking. At regular time intervals samples of 0.1 cm^3 were taken. The samples were seeded onto the surface of Petri dishes containing tributirin agar. The Petri dishes were stored overnight in a dark to avoid photo reactivation. Then the inoculated Petri dishes were cultivated at 30°C for 7 days. The number of survived colonies was counted and the survival curves were drawn. Screening of prospective mutant strains was carried out on the basis of the coefficient *K*, as described above. The screened fungal strains were isolated as pure cultures on PDA and stored at 4°C before further experiments.

Conditions for N-methyl-N-nitro-N-nitrosoguanidine mutagenesis (NMG)

Spore suspension containing 1.5x10^5 conidia cm^{-3} obtained from 7 days old culture of parental strain cultivated on PDA was treated with NMG dissolved in 0.05M citric buffer (pH 5.0) at concentrations of 100 μg cm^{-3} and 200 μg cm^{-3} for 6 h. The mutagenic effect of NMG was eliminated by adding 0.01% cystein. At regular intervals 0.1 cm^3 were taken. The samples were seeded onto the surface of Petri dishes containing tributirin agar. Screening of prospective mutant stains was carried out as listed above.

Conditions for submerged production of extracellular lipase

A nutritive medium of a following composition (g dm^{-3}): olive oil- 10, yeast extract- 5, peptone- 20, $MgSO_4.7H_2O$- 0.5, $ZnSO_4.7H_2O$- 0.4, $FeSO_4.7H_2O$- 1 and $MnSO_4$- 0.2, pH 7.0 was used for the submerged lipase production. The nutritive medium was sterilized at 121°C for 15 min. The cultivation was carried out in 300 cm^3 Erlenmeyer flasks, containing 30 cm^3 nutritive medium on a rotary shaker at 220 rpm, 30°C for 120 h. The nutritive medium was inoculated with 1% spore inoculum containing 10^9 conidia cm^{-3}. After the cultivation, the fungal biomass was separated through filtration and the cell free cultural broth was used as crude enzyme.

The amount of the biomass was determined gravimetrically by drying the samples to constant weight at 105°C. pH was determined potentiometrically using a pH-meter (Vario pH Stat, WTW, Germany).

Lipase assay

Lipase activity was assayed by alkali titration method using emulsified olive oil as a substrate (17). Ten cm^3 of olive oil was emulsified with 5% gum arabic (w/v) in 0.2M sodium phosphate buffer pH 7.0, using a domestic blender. The reaction mixture consisted of 9.0 cm^3 substrate emulsion and 1.0 cm^3 of crude enzyme. The enzyme reaction was carried out at 37°C for 10 min at gentle shaking at 150 rpm. The reaction was stopped by addition of 10.0 cm^3 acetone/ethanol solution 1:1 (v/v). The amount of fatty acids released was determined by titration with 0.05M NaOH using 1.0% (w/v) thymolphthalein as indicator. Blank samples were run in the same way by adding acetone/ethanol solution before the addition of the crude enzyme sample. One unit of lipase activity was defined as the amount of enzyme that releases 1.0 μmol fatty acids per min per cm^3 under the above assay conditions.

Correlation analysis

Correlation coefficient α that reflects the correlation between the coefficient *K* and the lipase activity determined in cell free cultural broth were calculated using Excel software at n=3.

All the experiments were carried out in triplicate and mean values are presented.

Results and Discussion

Twelve prospective microbial strains that belonged to 9 different fungal species were screened for the production of extracellular lipase. To evaluate the accuracy and effectiveness of the applied screening technique, lipase activity of all strains was also determined in submerged fermentation and the correlation between the screening coefficient *K* and the lipase activity were calculated.

As seen (**Table 1**), all the tested fungi exhibited extracellular lipase activity. The high values of the α coefficients demonstrated the reliability and the accuracy of the applied screening technique. Among the tested fungi *A. niger* displayed the highest lipase activity (7.5 U cm^{-3}) and was screened as parental strain for mutagenesis.

Usually strain improvement of enzyme producing filamentous fungi is reached by induced mutagenesis with various mutagens, such as UV light, N-methyl-N-nitro-N-nitrosoguanidine (NMG) and HNO_2, applied separately or in combination (2, 4, 5, 8, 12, 26). The effectiveness of the induced mutagenesis depends on the type of mutagen, dose and duration of exposition. As known, 99.9% kill correlates with the highest frequency of mutations and highest possibility for isolation of mutants with increased enzyme productivity (2, 8, 11, 12). Therefore, survival curves after UV and NMG treatment of *A.*

niger were drawn and analyzed (**Fig. 1** and **Fig. 3**). Survival less than 1.0% was reached for 160 s exposition on UV light. All of the survived 60 colonies were morphologically identical according to hyphal diameter, mycelium colour, diameter and colony shape, but only 15 colonies (25%) demonstrated lipase activities higher than the parental one (**Fig. 2**). Mutant strain UV_2 demonstrated the highest lipase activity (8.85 U cm^{-3}), which is 19% higher than the parental activity. The strain UV_2 kept its lipase activity unchanged in 5 consecutive generations and was screened as the most prospective UV mutant for further investigations. The achieved effect of UV mutagenesis on lipase activity of *A. niger* is almost equal with those achieved by Karaman and Medicherla (24) and lower than those achieved by Ellaiah et al. (18).

TABLE 1.

Screening of prospective extracellular lipase producing fungi

Fungal species	***K***	**Lipase activity (U cm^{-3})**	***α***
Aspergillus awamori	2.2	6.1	0.98
Aspergillus niger	3.7	7.5	0.98
Aspergillus oryzae	2.9	6.5	0.92
Aspergillus sp.1	2.1	6.0	0.92
Aspergillus sp.2	1.9	5.85	0.87
Aspergillus sp. 3	1.7	5.5	0.94
Aspergillus sp.4	1.4	5.2	0.92
Penicillium chryzogenum	2.4	6.2	0.92
Trichoderma atroviride	0.8	1.8	0.87
Trichoderma longibrachiatum	1.6	5.5	0.94
Trichoderma viride	1.7	5.6	0.98
Mucor sp.	2.1	6.2	0.98

Spore suspension of parental strain *A. niger* was subjected to mutagenesis with NMG at two concentrations: 100 and 200 μg cm^{-3}. The survival curves after NMG treatment are shown in **Fig. 3**.

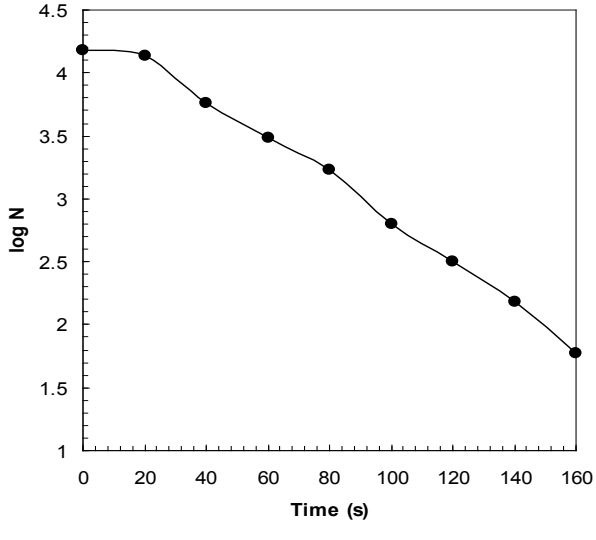

Fig. 1. Survival curve of *A.niger* after UV mutagenesis

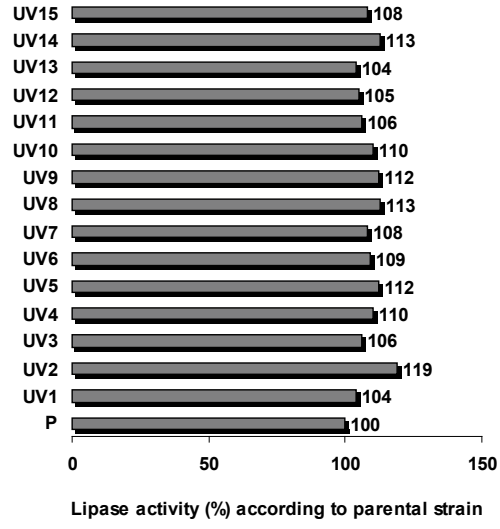

Fig. 2. Influence of UV treatment on lipase activity of *A. niger* P: Parental strain; UV: UV induced mutants

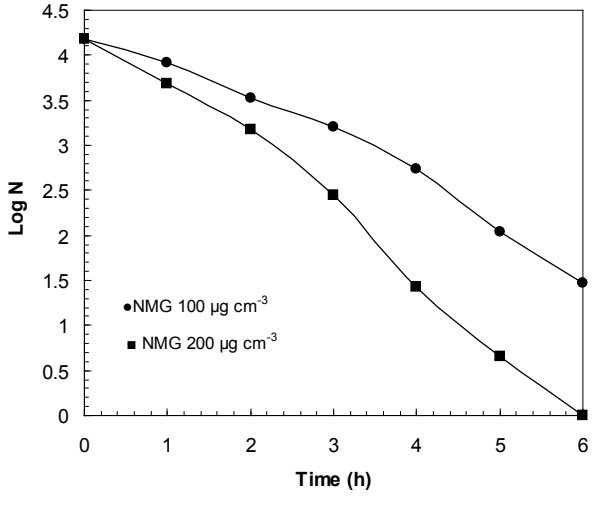

Fig. 3. Survival curves of *A.niger* after NMG mutagenesis

At NMG concentration of 100 μg cm^{-3} 0.2% survival was reached for 6 h. At NMG concentration of 200 μg cm^{-3} 0.18% survival was reached for 4 h. A total of 20% from the colonies survived after NMG treatment at concentration of 100 μg cm^{-3} (form NMG_1 to NMG_6) and 30% of the colonies survived after NMG treatment at concentration of 200 μg cm^{-3} (from NMG_7 to NMG_{15}) demonstrated lipase activities higher than the parental activity (**Fig. 4**). NMG concentration of 200 μg cm^{-3} was preferable in comparison to the concentration of 100 μg cm^{-3}, because low survivor and higher number of mutants with increased lipase activity were reached for shorter exposition time. All of the survived colonies were morphologically identical according to hyphal diameter, mycelium colour, and colony shape, but differed in colony diameter. According to the diameter, survived colonies were divided into two groups: small and big colonies. On the 7th day the small type colonies reached a diameter between 10 and 20 mm and the second

group of big type colonies reached a diameter between 21 and 31 mm. All of the strains which demonstrated increased lipase activities in comparison with parental strain were isolated from the big type colonies. Probably the utilization of oleaginous substrates from the nutritive medium as carbon sources favours the increase of the lipase activity. Therefore, great colony diameter was reached. Mutant strain NMG_{12} demonstrated the highest lipase activity (9.9 U cm^{-3}), which is 32% higher than the parental activity of *A. niger*. The strain NMG_{12} kept its lipase activity unchanged in 5 consecutive generations and was screened as the most prospective NMG mutant for further investigations.

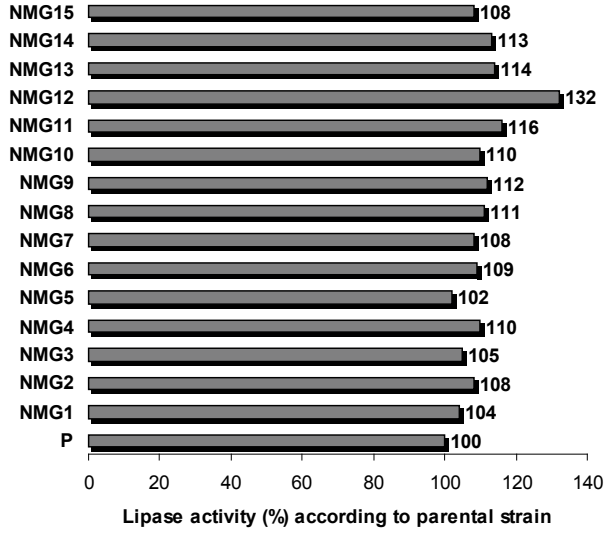

Fig. 4. Influence of NMG treatment on lipase activity of *A. niger* P: Parental strain, NMG: NMG induced mutants

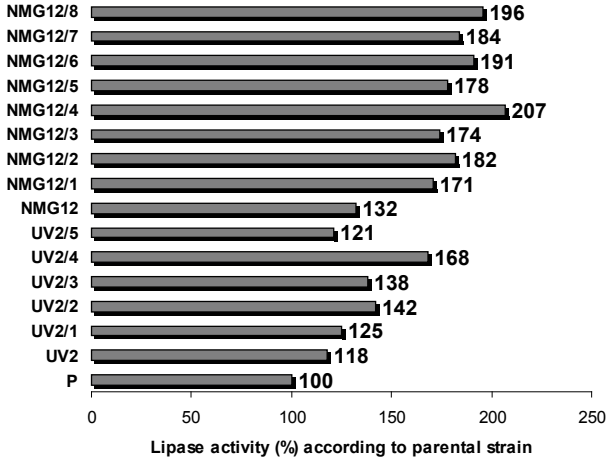

Fig. 5. Influence of the second step of NMG treatment on lipase activity of *A. niger* P: Parental strain; UV: UV induced mutants; NMG: NMG induced mutants

NMG caused higher increase of lipase activity in comparison with UV light and for this reason it was selected as more appropriate for the second step of mutagenesis. The selected mutants (UV_2 and NMG_{12}) were subjected to further mutagenic treatment with NMG at a concentration of 200 μg cm^{-3} for 4 h.

The second step of the mutagenic treatment with NMG increased additionally the lipase activity of the mutants. Five of the survived UV mutants after NMG treatment and 8 of the NMG mutants treated with NMG for the second time demonstrated higher lipase activities in comparison with the parental strains (**Fig. 5**).

All NMG mutants were more active in comparison with the UV mutants. The lipase activity of the best mutant from the UV set $UV_{2/4}$ was 12.6 U cm^{-3}, which is 68% higher in comparison with the parental strain *A. niger* (7.5 U cm^{-3}). The lipase activity of the best mutant from the NMG set $NMG_{12/4}$ was 15.5 U cm^{-3}, which is more than two times higher than the activity of the parental strain *A. niger* (7.5 U cm^{-3}). The mutant $NMG_{12/4}$ was selected for further investigations.

The dynamics of the extracellular lipase production by the selected mutant *A. niger* $NMG_{12/4}$ was also studied (**Fig. 6**). The maximum lipase activity (15.5 U cm^{-3}) was reached at 96 h, which corresponded to the end of the exponential growth phase. pH was slightly declined.

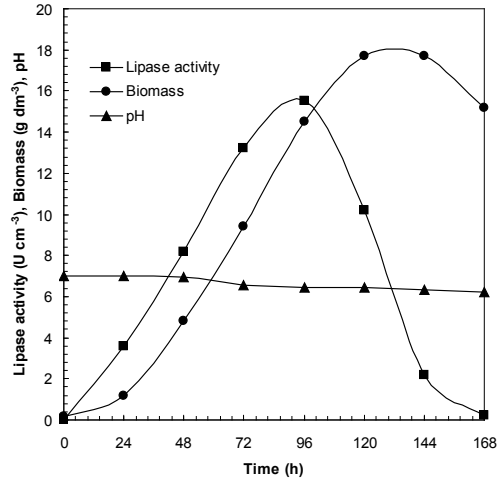

Fig. 6. Dynamics of extracellular lipase production by mutant *A. niger* $NMG_{12/4}$

The selected mutant strain was competitive and exceeded extracellular lipase productivity of other published fungal producers (1, 13, 16, 19, 23), even without optimization of the nutritive medium compositions and cultivation parameters.

Conclusions

Twelve microbial strains belonging to 9 different fungal species were screened for extracellular production of lipase. The most active lipase producing strain *A. niger* was selected for strain improvement by induced mutagenesis with UV light and NMG. It was determined that chemical mutagenesis was more effective in comparison with physical mutagenesis. By two stages mutagenesis with 200 μg cm^{-3} NMG for 4 h, lipase activity of parental strain was enhanced more than two

times. The selected mutant *A. niger* $NMG_{12/4}$ was stable and maximum lipase activity of 15.5 U cm^{-3} was reached at 96 h, which corresponded to the end of the exponential growth phase. The selected mutant is prospective for the development of industrial biotechnology for production of extracellular lipase. Then biodiesel production could be economically attractive. Experiments for optimization of nutritive medium composition and cultivation conditions are currently in progress.

REFERENCES

1. **Adinarayana K., Bapi Raju K.V.V.S.N., Zargar M.I., Devi R.B., Lakshmi P.J., Ellaiah P.** (2004) Ind. J. Biotechnol., **3**, 65-69.
2. **Bapi Raju K.V.V.S.N., Sujatha P., Ellaiah P., Ramana T.** (2004) African J. Biotechnol., **3**, 618-621.
3. **Chahinian H., Vanot G., Ibrik A., Rugani N., Sarda L., Comeau L.C.** (2000) Biosci. Biotechnol. Biochem., **64**, 215-222.
4. **Dacheva V. and Atev A.** (2005) Sci. Works UFT, **52**(2), 374-380.
5. **Ellaiah P., Prabhakar T., Ramakrishna B., Taleb A.T., Adinarayana K.** (2002) Indian J. Microbiol., **42**, 151-153.
6. **Fjerbaek L., Christensen K.V., Norddahl B.** (2009) Biotechnol. Bioeng., **102**, 1298-1315.
7. **Fukuda H., Kondo A., Noda H.** (2001) J. Biosci. Bioeng., **92**, 405-416.
8. **Gochev V. and Krastanov A.** (2007) Ecology and Safety Int. Sci. Publ., **1**, 260-267.
9. **Hasan F., Shah A., Hameed A.** (2009) Biotechnol. Adv., **27**, 782-798.
10. **Hiol A., Jonzo M.D., Rugani N., Druet D., Sarda L. Comeau L.C.** (2000) Enzyme Microb. Technol., **26**, 421-430.
11. **Hopwood D.A., Bibb M.J., Chater K.F, Kieser T., Bruton C.J., Kieser H.M., Lydiate D.J., Smith C.P., Ward J.M., Schrempf H.** (1985) Genetic manipulation of streptomyces, A laboratory manual, John Innes Foundation, Norwich, UK.
12. **Karanam S.K. and Medicherla N.R.** (2008) African J. Biotechnol., **7**, 2064-2067.
13. **Kashmiri M.A., Adnan A., Butt B.W.** (2006) African J. Biotechnol., **5**, 878-882.
14. **Lima V.M.G., Krieger N., Sarquis M.I.M., Mitchell D.A., Ramos L.P., Fontana J.D.** (2003) Food Technol. Biotechnol., **41**, 105-110.
15. **Martinelle M. and Hult K.** (1995) Biochim. Biophys. Acta, **1251**, 191-197.
16. **Pinheiro T.L.F., Menoncin S., Domingues N., Oliveira D., Treichel H., Di Luccio M.** (2008) Ciencia e Tecnologia de Alimentos, **28**, 444-450.
17. **Pinsirodom P. and Parkin K.L.** (2001) In: Current Protocols in Food Analytical Chemistry (R.E. Wrolstad, T.E. Acree, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, D.M. Smith, P. Sporns, Eds.), John Wiley and Sons, Inc., C3.1.1-C3.1.13.
18. **http**://www.nshtvn.org/ebook/molbio/Current%20Protocols/CPFAC/fac0301.pdf (accessed on 21. 01. 2011)
19. **Rajesh E.M., Arthe R., Rajendran R., Balakumar, Pradeepa C.N., Anitha S.** (2010) EJEAFChe, **9**, 1177-1189.
20. **Rani C. and Panneerselvam A.** (2009) ARPN J. Agricult. Biol. Sci., **4**, 39-43.
21. **Rigo E., Nonow J.L., Polloni A.E., Remonato D., Arbter F., Vardanega R., Oliveira D., Treichel H., Luccio M.** (2009) Industrial Biotechnol., **5**, 119-126.
22. **Sharma S., Chisti Y., Banerjee U.C.** (2001) Biotechnol. Adv., **19**, 627-662.
23. **Shukla P. and Gupta K.** (2007) J. Appl. Sci. Environ. Sanit., **2**, 35-42.
24. **Teng Y. and Xu Y.** (2008) Biores. Technol., **99**, 3900-3907.
25. **Treichel H., Oliviera D., Mazzutti M.A., Luccio M., Oliviera J.V.** (2010) Food Process. Technol., **3**, 182-196.
26. **Vardanega R., Remonatto D., Arbter F., Polloni A., Rigo E., Ninow L.N., Treichel H., Oliveira D., Luccio M.** (2010) Food Process. Technol., **3**, 461-465.