

Toxicity of some solvents and extractants towards *Lactobacillus casei* cells

N. A. Marinova, D. S. Yankov*

*Institute of Chemical Engineering, Bulgarian Academy of Sciences,
Acad. G. Bonchev St., Block 103, 1113 Sofia, Bulgaria*

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Various organic compounds used as diluents, modifiers and extractants in lactic acid extraction have been tested to determine their toxicity towards *Lactobacillus casei* cells. The toxicity on molecular and phase level has been investigated. In general, the tested hydrocarbons were non-toxic on molecular level and showed variable toxicity on phase level. Among the tested alcohols octanol and oleyl alcohol were non-toxic, whereas decanol and dodecanol were toxic on the molecular level. All the alcohols showed high phase toxicity. The tested extractants were very toxic both on molecular (except for tridodecylamine) and on phase level. Two extraction systems composed of trioctylamine/oleyl alcohol and tridodecylamine/oleyl alcohol have also been studied. Both systems were non-toxic on molecular level and showed medium toxicity on phase level. They can be used successfully for *in situ* extractive fermentation of lactic acid.

Key words: solvent toxicity, *in-situ* extraction, lactic acid, *Lactobacillus casei*.

INTRODUCTION

The product separation or *in situ* product removal is the limiting stage in the manufacturing of various organic compounds, produced via microbial transformations, because of low concentration and water solubility of the product. The product recovery step is very expensive and energy consuming. The fermentative lactic acid production is a typical example of an end-product inhibited process. Besides, the rate decreasing due to inhibition, accumulation of the acid leads to decrease in pH value of the system, often out of the optimum range for fermentation. In case of such fermentations processes where the continuous removal of the product is obligatory, successful organization of the process is very complicated. Different approaches, such as solvent extraction [1, 2], electro-dialysis [3, 4], membrane separation [5, 6], ion-exchange [7, 8] or aqueous two-phase systems [9] have been used for overcoming the arising problems. Each one of above mentioned methods possesses its own advantages and draw-backs.

Solvent extraction, especially extraction with long-chain tertiary aliphatic amines, has been recognized as a promising alternative to conventional calcium-salt precipitation method for separation and purification of the lactic acid. The major problem in this case is the toxicity of the commonly used organic solvents. The mechanism of toxicity caused by the organic solvents is still not clear, but it is generally accepted that the interaction of the

solvents with membrane lipids leads to disturbance of essential membrane functions, inactivation or denaturation of membrane-bound enzymes, breakdown of transport mechanisms and at high concentrations to solvolysis of the cells [10–12]. The solvents interact with the cells by two routes: direct contact of the cells with the water immiscible organic phase and by solvent molecules dissolved in fermentation broth. The former mechanism is named “phase” toxicity and the latter – “molecular” toxicity.

Chen and Lee [5] have investigated the toxicity of three diluents, three modifiers and 5 solvent mixtures towards *Lactobacillus delbrueckii* strain. They have concluded that kerosene and oleyl alcohol are nontoxic and have chosen the system composed of 20% Alamine 336, 40% kerosene and 40% oleyl alcohol for *in situ* extraction of lactic acid.

Seevaratnam *et al.* [13] have reported that Aliquat 336, Amberlite LA-2, Adogen 464 and trioctyl amine (TOA) are highly toxic towards *Lactobacillus delbrueckii* cells even at 0.1% concentration. What is more, Aliquat 336 and Adogen 464 formed third phase during extraction, when dissolved in paraffin oil in order to improve their physical properties.

Tong *et al.* [14] have studied 10 extractants and solvents in regard to their toxicity to *Lactobacillus rhamnosus* cells and have used a mixture of TOMAC (trioctylmethylammoniumchloride, 0.2 kmol/m³) in oleyl alcohol, in spite of the high toxicity of TOMAC.

* To whom all correspondence should be sent:
E-mail: yanpe@bas.bg

Demirci *et al.* [15] have made a screening of 12 solvents and 8 carrier compounds in different combinations, with respect to their toxicity on *Lactobacillus casei* strain. Hexadecane:tributyl phosphate, *n*-dodecane:tri-*n*-octylamine, and kerosene:tri-*n*-octylphosphine oxide demonstrated the least microbial toxicity among the tested blends with excess solvent media. Whereas hexanes:Alamine 304 and xylenes:Alamine 304 were non-toxic in solvent saturated media.

Choudhury *et al.* [16] have also investigated the toxicity of TOA in three different solvents (methylisobutyl ketone (MIBK), octanol, paraffin) on *Lactobacillus delbrueckii* strain. While TOA was found to be highly toxic on the molecular and on the phase level, the paraffin liquid was totally non-toxic.

The data presented in the literature varied and they are often contradictory. It seems that the toxicity of organic solvents and extractants is closely related to the type of used microorganism.

The aim of the present study was to investigate the solvent toxicity towards *Lactobacillus casei* strain of various organic solvents and extractants, frequently used for extraction of lactic acid from fermentation broth.

EXPERIMENTAL

Materials and Methods

Extractants. Tributylphosphate (TBP) – Merck; Tridodecylamine (TdDA) – Fluka; Dioctylamine (DOA) – Fluka; Tri-*n*-octylamine (TOA) – Acros Organics; Alamine 336 (a mixture of tri-*n*-octylamine and tri-*n*-decylamine) – Henkel and Aliquat 336 (tri(C₈C₁₀)methylammonium chloride) – Acros Organics.

Modifiers. 1-Octanol – Fluka; 1-decanol – Merck; 1-dodecanol – Merck; oleyl alcohol – Acros Organics.

Diluents. *n*-Octane – Fluka; *n*-Decane – Merck; Dodecane – Prolabo; kerosene – technical grade, distillation fraction from 175–190°C with $\rho^{20} \sim 0.778 \text{ kg/dm}^3$.

All chemicals, except for kerosene, were p.a. purity grade. Kerosene was distilled from a technical grade, taking the 198–212°C fraction. Before further use, the organic chemicals were washed threefold with distilled water under vigorous mixing for 10 min in order to eliminate any water-soluble impurities. At the same time, the organic chemicals were saturated with water.

Microorganism. *Lactobacillus casei* strain (NBMCC-1013) was used in the present study.

Media. The strain was maintained on a semi-synthetic medium containing (g/l): yeast extract 10; peptone 10; sodium acetate 5; MgSO₄·7H₂O 0.1; MnSO₄·4H₂O 0.05; agar 20; distilled water to 1l.

The culture was inoculated into semi-synthetic medium containing (g/l): yeast extract 5.5; peptone 12.5; KH₂PO₄ 0.25; K₂HPO₄ 0.25; sodium acetate 10.0; MgSO₄·7H₂O 0.1; MnSO₄·4H₂O 0.05; FeSO₄·7H₂O 0.05; distilled water to 1l. The pH value was adjusted to 6.8. The culture media were sterilized at 121°C for 20 min. The bacterial cells were transferred from agar slants into the medium and were incubated for 24 h at 38°C in a rotatory shaker New Brunswick Scientific Co., Ink., Edison, NY, USA, (100–120 rpm). In a conventional fermentation process, 10 ml of the inoculum were added to 100 ml of the culture medium. The fermentation was carried out in flasks without any pH correction for 48 h.

Molecular level toxicity experiments. Ten millilitres of washed chemicals were mixed with 100 ml of distilled water and were shaken for 15 min in a shacking machine. After phase separation, the solvent saturated water was used for culture medium preparation. After fermentation without any pH control, samples were taken at 24 h and 48 h intervals and were analyzed for produced lactic acid and bacterial growth. The results were compared with those of control sample, where the medium was prepared with distilled water.

Phase level toxicity experiments. For these experiments water saturated organic solvents were sterilized and 25 ml of each one of them were added to 100 ml of culture media. The fermentation process was carried out as it was described above and the results were compared to the control sample.

Analysis

Counting of microbial cells. After appropriate dilution of the sample the number of the cells in one ml was counted with the help of a Bürker camera.

Lactic acid analyses. An HPLC system composed of Perkin-Elmer Series 10 Pump, LC-25RI detector, Shimadzu C-R6A Chromatopac integrator and Aminex HPX-87H column was used. The mobile phase was 0.01 N H₂SO₄ at 0.6 ml/min flow rate. Pure (98%) crystalline L-(+)-lactic acid (Sigma) was used as the standard.

RESULTS AND DISCUSSION

In order to divide the used organic compounds into groups according to their toxicity, the classification proposed by Martak *et al.* [17] was used:

- *Non-toxic solvents* when practically no toxic effect was observed and the production rate was at most 25% lower than in the control cultivation sample.

- *Solvents with medium toxicity* when the production rate was higher than 25% of that in control cultivation sample.

- *Toxic solvents*, when no biological activity was observed in their presence or the production rate was less than 25% of that in control cultivation sample.

Molecular level toxicity

Diluents. All the investigated hydrocarbons are non-toxic on molecular level. Both culture growth and lactic acid production are at least 80% of the control sample at 24 h and 48 h of fermentation. The results are represented in Figure 1.

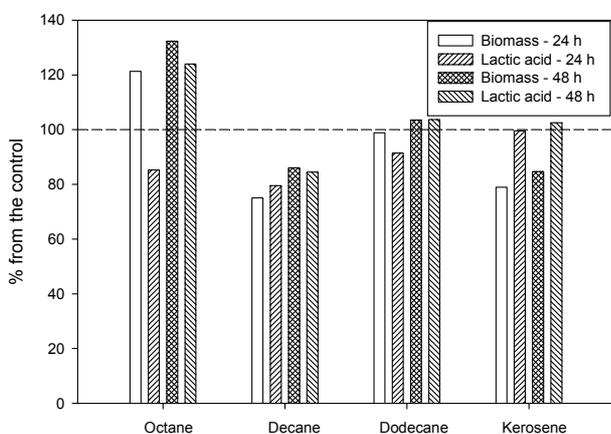


Fig. 1. Influence of some diluents on the cell growth and lactic acid production of *Lactobacillus casei* - molecular level.

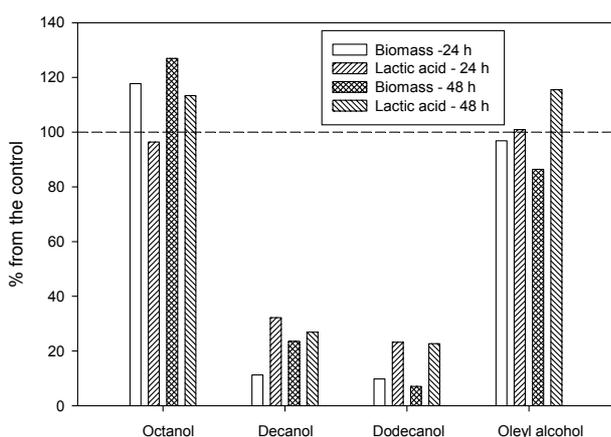


Fig. 2. Influence of some modifiers on the cell growth and lactic acid production of *Lactobacillus casei* - molecular level.

Dodecane and kerosene had the same behavior at two points of comparison, whereas the production of lactic acid in case of octane increased at 48 h and

decreased in the presence of decane.

Modifiers. The toxicity of the investigated alcohols differs considerably. The octanol and oleyl alcohol are non-toxic and their results are close to the control sample ones. The bacterial growth (11% and 10% at 24 h; 24% and 23% at 48 h) and the lactic acid production (32% and 23% at 24 h and 23% and 7% at 48 h) for decanol and dodecanol were very low. The results obtained are shown in Figure 2.

Extractants. Among all extractants used, only tridodecylamine is not-toxic on molecular level (Fig. 3).

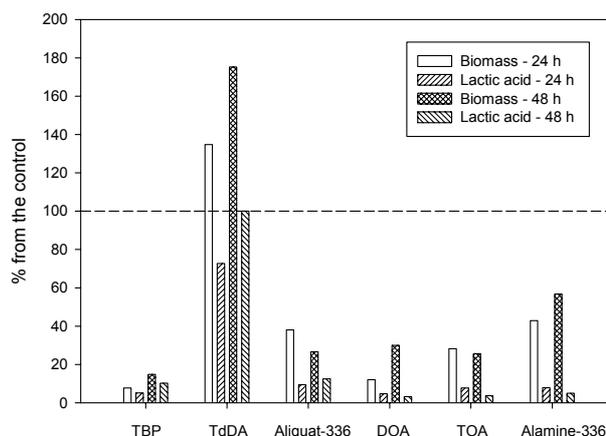


Fig. 3. Influence of some extractants on the cell growth and lactic acid production of *Lactobacillus casei* - molecular level.

What is more the number of the cells is greater than in the control sample (135% at 24 h and 175% at 48 h). It is necessary to mention that the bacterial cells look differently. They look not like small sticks, but like circles. If a sample of these cells is inoculated in normal fermentation medium they restore their initial shape, as well as the growth rate and productivity. This phenomenon has to be additionally investigated. For all other extractants the number of the cells does not exceed 60% and the lactic acid produced – 15%.

Phase level toxicity

Diluents. The results obtained (Fig. 4) demonstrate considerable phase toxicity of the investigated hydrocarbons, regardless of the high number of the cells. In case of octane and decane the number of the cells is 2 to 4 time higher than in the control sample. Again the cells look like small circles and do not have their usual shape. The increased number of the cells does not result in increase of the lactic acid production. The lactic acid production in the presence of octane and decane (compared to the control sample) is 11% and 21% at 24 h and 42%

and 33% at 48 h respectively. The dodecane is non-toxic on the phase level, whereas the kerosene fraction is very toxic – (25% and 33% biomass, 14% and 13% lactic acid).

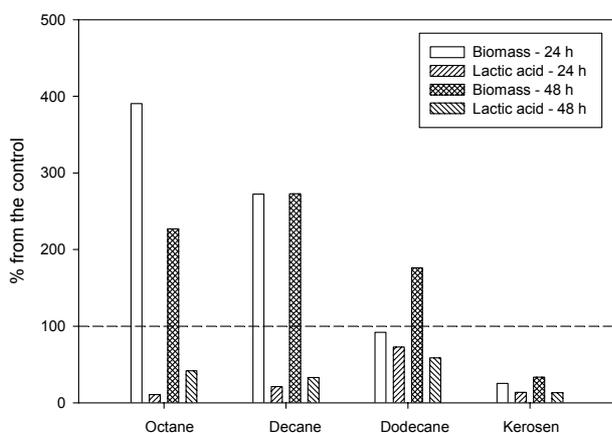


Fig. 4. Phase level toxicity of some diluents on *Lactobacillus casei* cells.

Modifiers. Similarly to the hydrocarbons, the corresponding alcohols (octanol and decanol) showed high phase toxicity. In the case of octanol, on the 24 h, the biomass is only 2% of the control sample, increasing slightly to 8% at 48 h – (Fig. 5).

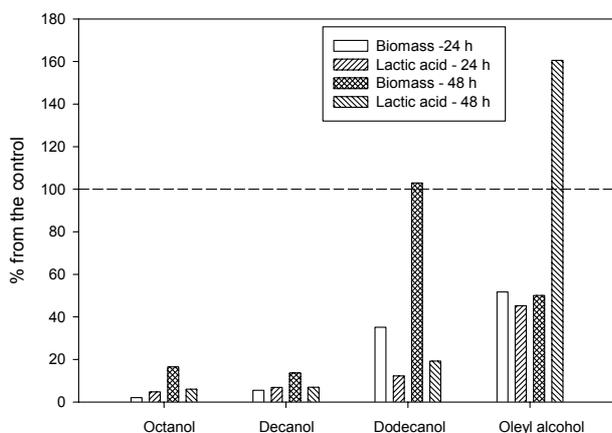


Fig. 5. Phase level toxicity of some modifiers on *Lactobacillus casei* cells.

The results for lactic acid production are analogous – 5% at 24 h and 7% at 48 h. For decanol we obtained - 6% and 7% biomass at the 24 h and 48 h and 7% lactic acid for both points of analysis. The results for the dodecanol are very different comparing the biomass growth and lactic acid production. Whereas at the 24 h the biomass is only about 35% of the control sample, at the 48 h the biomass is equal to that of the control sample. At the same time the production of the lactic acid is low – 12% and 19%. Probably in this case the acid production has started at the late stage of fermentation, because the cells were viable and with

normal shape. In the case of oleyl alcohol the phase toxicity is low. In spite of the fact that the biomass is only about 50% at the 24 h and 48 h, at the end of the fermentation, the produced lactic acid is 160% compared to the control sample.

Extractants. All the studied extractants showed high phase level toxicity. The number of the cells did not exceed 45% of the control sample and produced lactic acid – 11% (Fig. 6).

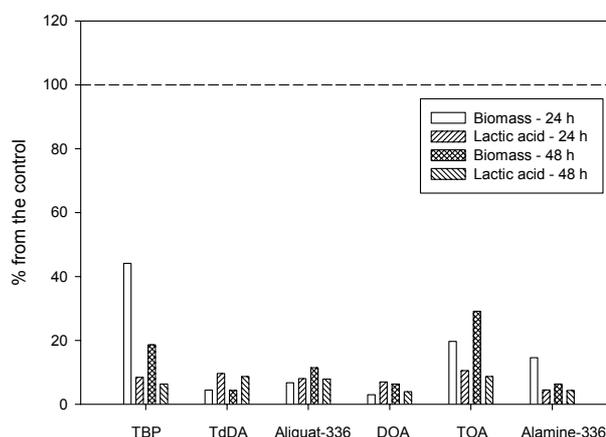


Fig. 6. Phase level toxicity of some extractants on *Lactobacillus casei* cells.

The lactic acid was analyzed only in water phase and we had no information for the quantity of the extracted acid. This will be done on the next stage of the investigations. In any case, if there is some extracted acid the results will be better. At the same time the cells have normal shape and it is possible that the presence of organic phase prolonged the lag phase. The results obtained at two points of comparison do not give enough information about the cell growth in the presence of organic reagents. Additional investigations are necessary and the fermentation process should be monitored at shorter time intervals during entire process.

Systems extractant/modifier. On the basis of the results obtained it has been decided to check two systems, composed of TOA/oleyl alcohol and TdDA/oleyl alcohol at volume ratio 30/70.

On the molecular level both systems showed no toxicity. The results at 24 h are very close to those of the control sample and decreased to about 70% for the biomass and 60% for lactic acid at 48 h (Fig. 7a). The results with TdDA/oleyl alcohol system are similar to those with pure TdDA, whereas the system TOA/oleyl alcohol is less toxic than the pure TOA.

On the phase level of toxicity the chosen systems are better, compared to the pure extractants. The cell growth in the system TOA/oleyl alcohol is about 70% of the control sample and in the system TdDA/oleyl alcohol it is even higher than that in the

control sample (Fig. 7b). The produced lactic acid is about 25% for both systems, ignoring the quantity of acid extracted by the amine.

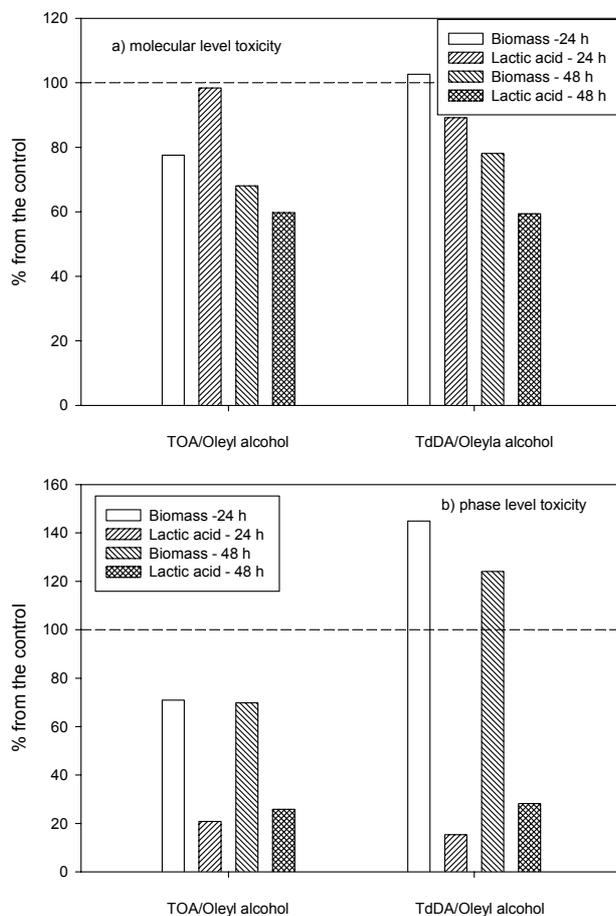


Fig. 7. Molecular (a) and phase level (b) toxicity of the systems TOA/oleyl alcohol and TdDA/oleyl alcohol on the *Lactobacillus casei* cells.

CONCLUSIONS

Among all the tested diluents and modifiers only decanol and dodecanol were toxic on molecular level, as well as all the extractants, except for tridodecylamine. All other chemicals were non-toxic on molecular level.

Regarding phase level toxicity, only dodecane, dodecanol and oleyl alcohol showed low toxicity, while all the extractants were highly toxic.

The results obtained with systems extractant/diluent demonstrated that the use of non-toxic diluents decreases the toxicity of the system in comparison to pure extractants.

Further investigations are necessary to determine whether lower lactic acid production is due to the prolonged lag-phase in bacterial growth, as well as on the influence of ratio extractant to diluent on the lactic acid production.

REFERENCES

1. K. Ye, Sh. Jin, K. Shimizu, *J. Ferment. Bioeng.*, **81**, 240 (1996).
2. N. Tik, E. Bayraktar, Ü. Mehmetoglu, *J. Chem. Techn. Biotechnol.*, **76**, 764 (2001).
3. L. Xuemei, L. Jianping, L. Mo'e, C. Peilin, *Bioprocess Eng.*, **20**, 231 (1999).
4. K.B. Lee, *Bioresource Technol.*, **96**, 1505 (2005)
5. S. R. Chen, Y. Y. Lee, *Appl. Biochem. Biotechnol.*, **63-65**, 435 (1997).
6. Y. Tong, M. Hirata, H. Takanashi, T. Hano, F. Kubota, M. Goto, F. Nakashio, M. Matsumoto, *J. Membrane Sci.*, **143**, 81, (1998).
7. A. V. Sosa, J. Ochoa, N. I. Perotti, *Bioseparation*, **9**, 283 (2001).
8. W.-Y. Tong, X.-Y. Fu, S.-M. Lee, J. Yu, J.-W. Liu, D.-Zh. Wei, Y.-Mo Koo, *Biochem. Eng. J.*, **18**, 89 (2004).
9. J. Planas, A. Kozłowski, J. M. Harris, F. Tjerneld, B. Hanh-Hagerdal, *Biotechnol. Bioeng.*, **66**, 211 (1999).
10. P. Nikolova, O. P. Ward, *J. Ind. Microb.*, **12**, 76 (1993).
11. R. Leon, P. Fernandes, H. M. Pinheiro, J. M. S. Cabral, *Enz. Microb. Techn.*, **23**, 483, (1998).
12. Y. Sardesai, S. Bhosle, *Res. Microbiol.*, **153**, 263 (2002).
13. S. Seevaratnam, S. Holst, O. Hjørleifsdottir, B. Mattiasson, *Bioprocess Eng.*, **6**, 35 (1991).
14. Y. Tong, M. Hirata, H. Takanashi, T. Hano, M. Matsumoto, Sh. Miura, *Sep. Purif. Techn.*, **33**, 1439 (1998).
15. A. Demirci, A. L. Pometto III, K. R. Harkins, *Bioseparation*, **7**, 297 (1999).
16. B. Choudhury, A. Basha, T. Swaminathan, *J. Chem. Techn. Biotechnol.*, **73**, 111 (1998).
17. J. Marták, E. Sabolová, Š. Schlosser, M. Rosenberg, L. Křištofiková, *Biotechnol. Techn.*, **11**, 71 (1997).

ТОКСИЧНОСТ НА НЯКОИ РАЗТВОРИТЕЛИ И ЕКСТРАГЕНТИ КЪМ КЛЕТКИ НА *LACTOBACILLUS CASEI*

Н. А. Маринова, Д. С. Янков*

*Институт по инженерна химия, Българска академия на науките,
ул. „Акад. Г. Бончев”, бл. 103, 1113 София*

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(Резюме)

Различни органични съединения, използвани като разтворители, модификатори и екстрагенти при екстракцията на млечна киселина са изследвани, за да се определи токсичността им спрямо клетки на *Lactobacillus casei*. Изследвана бе токсичността на молекулно и фазово ниво. Изследваните въглеродороди са нетоксични на молекулно ниво и показват различна токсичност на фазово ниво. Измежду изследваните алкохоли октанолът и олеиловият алкохол са нетоксични, а деканолът и додеканолът са токсични на молекулно ниво. Всички алкохоли показват висока токсичност на фазово ниво. Изследваните екстрагенти са силно токсични както на молекулно (с изключение на тридодециламина), така и на фазово ниво. Две екстракционни системи, съставени от триоктиламин/олеилов алкохол и тридодециламин/олеилов алкохол също бяха изследвани. И двете системи са нетоксични на молекулно ниво и са средно токсични на фазово ниво. Те успешно могат да се използват за *in situ* екстрактивна ферментация на млечна киселина.