Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC
JOURNAL
OF POLISH
AGRICULTURAL
UNIVERSITIES

2001
Volume 4
Issue 2
Series
FOOD SCIENCE AND TECHNOLOGY

SIP A., GRAJEK W. 2001. TRANSFER OF DIVERCIN THROUGH ULTRAFILTRATION MEMBRANES AFTER TREATMENT WITH DETERGENTS AND ORGANIC SOLVENTS **Electronic Journal of Polish Agricultural Universities**, Food Science and Technology, Volume 4, Issue 2.
Available Online http://www.ejpau.media.pl

TRANSFER OF DIVERCIN THROUGH ULTRAFILTRATION MEMBRANES AFTER TREATMENT WITH DETERGENTS AND ORGANIC SOLVENTS

Anna Sip, Włodzimierz Grajek
Department of Biotechnology and Food Microbiology, Agricultural University of Poznań, Poland

ABSTRACT

The ultrafiltration of post-culture fluids proved that divercin occurred in the form of large macroaggregates with the molecular weight over 100 kDa. Addition of detergents: Tween 80, Nonidet P-40 and SDS, and alcohols: ethanol and isopropanol into supernatants from post-culture fluids caused partial disaggregation of divercin. The better effect was obtained when Tween 80 was introduced directly into culture medium. However, other detergents and both alcohols showed a toxic effect to the bacteria and reduced the divercin production in *C. divergens* culture. The ultrafiltration yield of divercin depended on the type of membranes and treatment of divercin-containing extracts with detergents and alcohols. The cellulose acetate membranes were more

permeable for divercin that made from polyethersulfone. The highest divercin disaggregation in active extracts, made in evidence by the improvement of divercin transfer through membrane cascade, was obtained as result of 1 % of SDS and Nonidet P-40 addition.

Key words: divercin, bacteriocin, ultrafiltration, detergents, alcohols, *Carnobacterium divergens*, aggregation, polyethersulfone membrane, cellulose acetate membrane

INTRODUCTION

In the last decade, bacteriocins are the subject of investigations of many scientific laboratories. It results from their biological activity and a great potential in food, medicine and environmental applications. Up-today the most known bacteriocin is nisin which is widely used in food industry to protect the products against the pathogenic and spoiling bacteria [1,3].

Many of bacteriocins contain the hydrophobic domains and tent to form large aggregates. Through these domains they can aggregate with lipids and other proteins presented in culture media or in food products [4,9,10,7,13,5]. This phenomenon leads to inactivation of bacteriocin and should be considered as disadvantageous.

The large macromolecules of bacteriocins can be disaggregated by the use of surface active compounds. The treatment of bacteriocins with detergents results in significant increase of bacteriocin activity [10,6]. High molecular aggregates can be also split by physical treatment, e.g. ultrasounds, high speed stirring, pumping and membrane filtration.

One of the more promising bacteriocin is divercin produced by lactic acid bacteria *Carnobacterium divergens* [12]. It show an antilisterial activity and high thermostability. Divercin is a 43-amino-acid peptide of 4.5 kDa with two disulfide bonds [11]. Preliminary studies suggested that both disulfide bonds and hydrophobic amino-acids are essential for antilisterial activity of this bacteriocin [8]. It is also interesting to note that divercin has three trytophan residues. These residues could be important for insertion in target membrames. Because of these hydrophobic domains this bacteriocin can also form large polymers. It limits its activity and application in food products containing lipids and hydrophobic proteins.

The aim of this investigation was to determine the effect of detergents and organic solvents on the aggregate formation by divercin in culture media and estimation of divercin transfer through the different types of ultrafiltration membranes.

MATERIALS AND METHODS

Microorganism and divercin production

For bacteriocin production the bacteria strain *Carnobacterium divergens* AS7 was used. Microorganism was cultivated on the modified MRS medium in which triptone was replaced with casein peptone. The medium was sterilised at 121°C for 60 min. Bacteria were cultured in Bioflo III bioreactors (New Bruswick Sci., Edison, N.J., USA) in optimum conditions for divercin production, i.e. at 30°C, at constant pH 6.5, stirring 80 rpm, in anaerobic conditions. The pH was kept constant by automatic injection of 5 M NaOH. Fresh media were inoculated with 2% v/v 14 h old culture of bacteria. The culturing was carried out for 15 h. In one experiment set the culture was performed in the medium with 1% Tween 80 addition.

Indicator bacteria *Carnobacterium piscicola* NCDO 2765, used in divercin activity determination, were cultured at 30°C in a culture medium containing 1% of glucose, 1% NaCl and 0.5% of yeast extract. [14].

Preparation of divercin active extracts

After the end of exponential phase of bacteria growth, the culture liquid was taken and centrifuged (10 min, 8,000 g). The obtained supernatant was heated for 10 min in 100°C in order to inactivate proteolytic enzymes, and then cooled in an ice water bath. The supernatant was subsequently referred to as an active extract.

Disaggregation of divercin with alcohols and detergents

Detergents Tween 80, Nonidet P-40 and SDS in the concentration of 0.01, 0.05, 0.1, 1% w/v as well as ethanol and isopropanol in the concentration of 1, 3, 5 and 7% w/v were introduced into divercin active extracts. The prepared extracts were incubated at 30°C for 4 hours while constantly stirring. After incubation, the solutions were subjected to cascade ultrafiltration through subsequent filters. The obtained retentates and permeates were tested in terms of their antimicrobial activity. In the determination of divercin activity, the control samples were those same extracts with divercin inactivated with pronase E (Serva), used in concentration of 0.1 mg/mL.

Ultrafiltration mode

Filtration of divercin active extracts was carried out using two filtration units: (1) Filtron model Minisette cross flow system equipped with cassette of polyethersulfone membranes of the Omega series with cut off 5, 10, 30, and 100 kDa, and (2) Amicon ultrafilter, model CH2RSA, equipped with spiral membranes of cellulose acetate with cut off 5, 10, 30, and 100 kDa. The both types of membrane were not protein binding. The filtration was carried out using filter cascade with gradually decreasing pore diameter from 100 kDa to 5 kDa.

1000 mL of divercin active extracts (filtration feed) were filtered by membranes and divercin activity was then determined in permeates. Control samples were those same active extracts with divercin inactivated with proteolytic enzyme Pronase E (Serva), used in concentration of 0.1 mg/mL.

The determination of divercin antibacterial activity

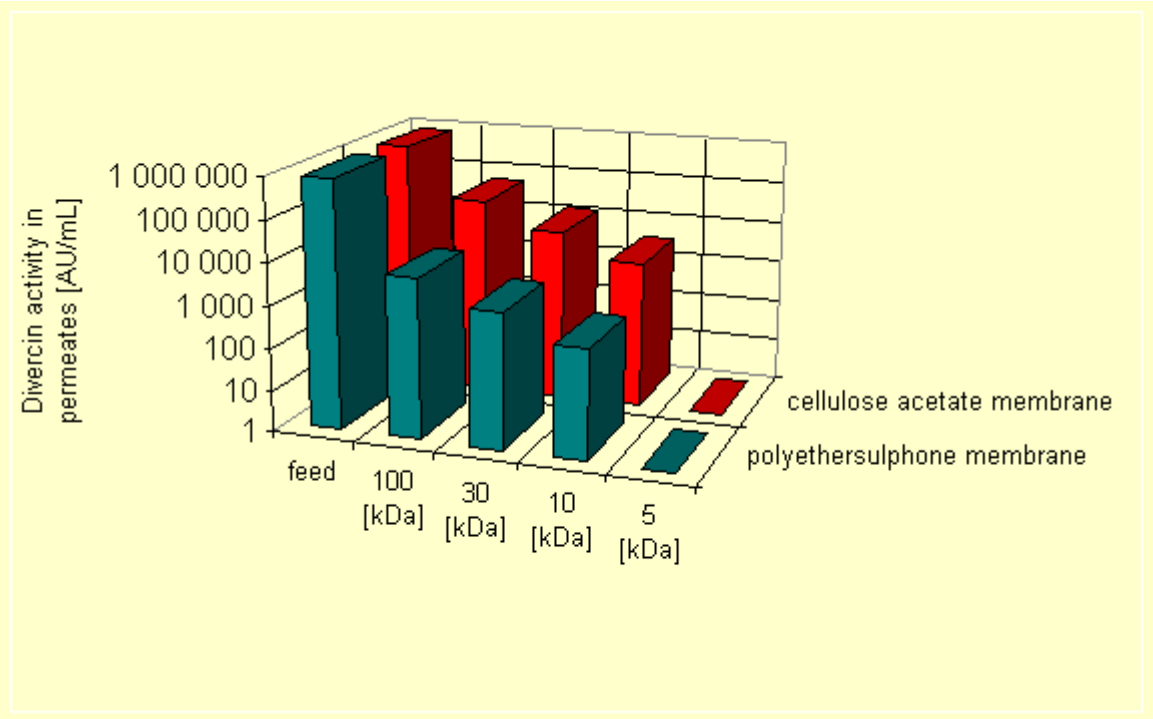
The activity of divercin was determined by the method of critical dilutions, described by Pilet et al. [12] in relation to indicator bacteria *Carnobacterium piscicola* NCDO 2762 (E.N.I.T.I.A.A., Nantes, France) and expressed in conventional units AU/mL, which denote the inverse of the lowest concentration of a sample which no longer exhibits the ability to inhibit the growth of indicator bacteria.

RESULTS AND DISCUSSION

The results of experiments confirm the tendency to form large macromolecules of divercin as an effect of bacteriocin monomer aggregation. Only a part of divercin activity occurring in active extract (filtration feed) was transferred into permeate after the first step of filtration through the 100 kDa membranes. It means that the dominant fraction of divercin present in the active extract obtained from bioreactor culture, occurred in the form of macromolecules with molecular weight over 100 kDa.

On the ground of the data obtained, it was found that the passage of active extract through an ultrafilter cascade resulted in gradually decrease of bactericidal activity of divercin solution. The highest reduction of divercin activity was detected in permeates obtained from 100 kDa membranes of the both types, whereas, the membranes with cut off 5 kDa were entirely impermeable to divercin molecules (Fig. 1).

Fig. 1. Transfer of divercin activity through cascade of ultrafiltration membranes of different types versus their decreasing pore diameters

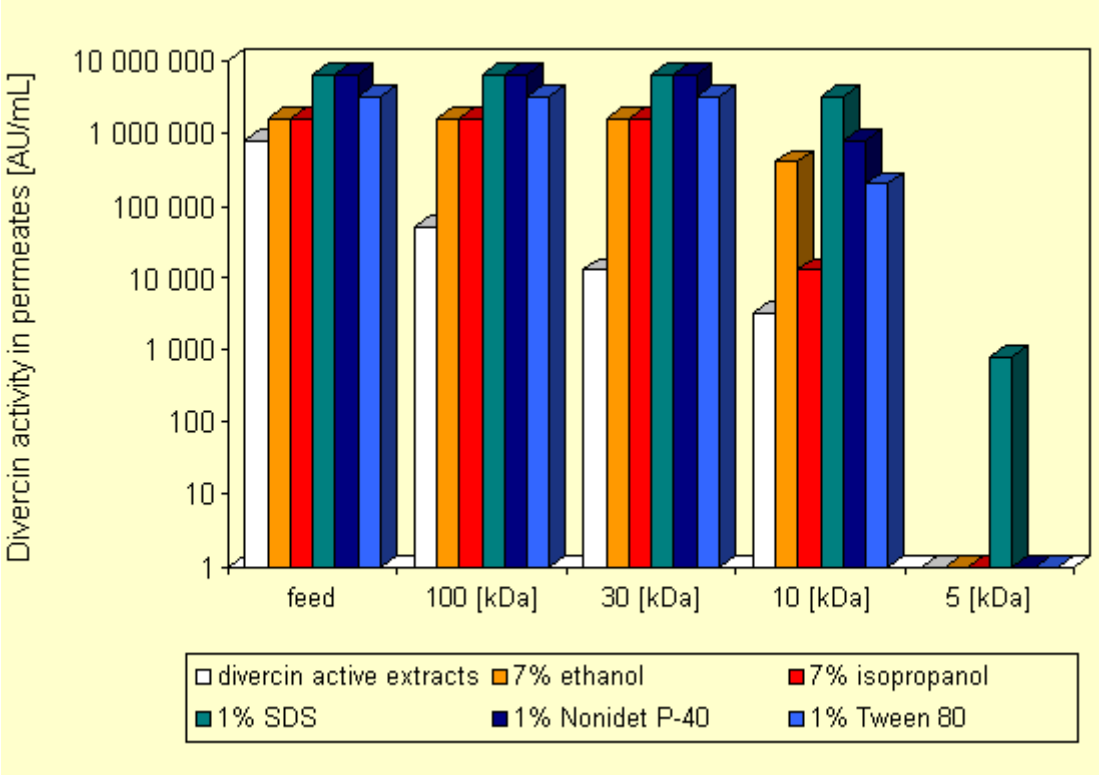


The results show that the divercin passage through membranes depends on the type of membrane used. Generally, it can be assumed that cellulose acetate membranes are more permeable to divercin molecules than the polyethersulfone membranes. In each permeate collected from polyethersulfone membranes the divercin activity was significantly lower then in those from cellulose acetate equivalents with adequate cut off. On the basis of this results it can be assumed that divercin has more afinity to the polyethersulfone membranes.

It should be stressed that divercin aggregation is disadvantageous because it make membrane filtration difficult as a method in bacteriocin separation and purification. It is also unfavorable in continuous divercin production using membrane bioreactor. On this ground, we undertake the study on divercin disaggregation using detergents and alcohols. Detergents and alcohols investigated in this study are usually used as a components of cleaning solutions. Divercin has a strong antilisterial activity and it is possible to use it as constituent of disinfectant solutions. As this application is concerned there is no need to remove detergents and alcohols from divercin extracts.

The completed experiments showed that addition of ethanol, isopropanol, Nonidet P-40, SDS and Tween 80 to active extract improved considerably divercin passage by membrane cascade with cut off 100 kDa, 30 kDa, 10 kDa and 5 kDa (Fig. 2).

Fig. 2. Effect of detergents and alcohols on the divercin activity passage through the cascade of cellulose acetate membranes versus their decreasing pore diameter



Divercin present in active extracts with 7% of ethanol and isopropanol addition as well as in active extract with 1% of Nonidet P-40, SDS and Tween 80 addition passed entirely through 100 kDa and 30 kDa membranes without any activity losses. However, it was partially stopped on the 10 kDa membranes. On this basis, it can be concluded that addition of mentioned chemicals in above defined concentrations did not caused the entire divercin disaggregation but split the bacteriocin macromolecules mainly to dimers. However, a small part of divercin activity, amounted 400 AU/mL, was detected in permeates from 5 kDa polyethersulfone membrane when 1% of SDS was added and in permeates from 5 kDa cellulose acetate membrane when 1% of SDS and 1% of Tween 80 was added, with activity of 800 AU/mL respectively. It means that these both detergents can split divercin to monomers.

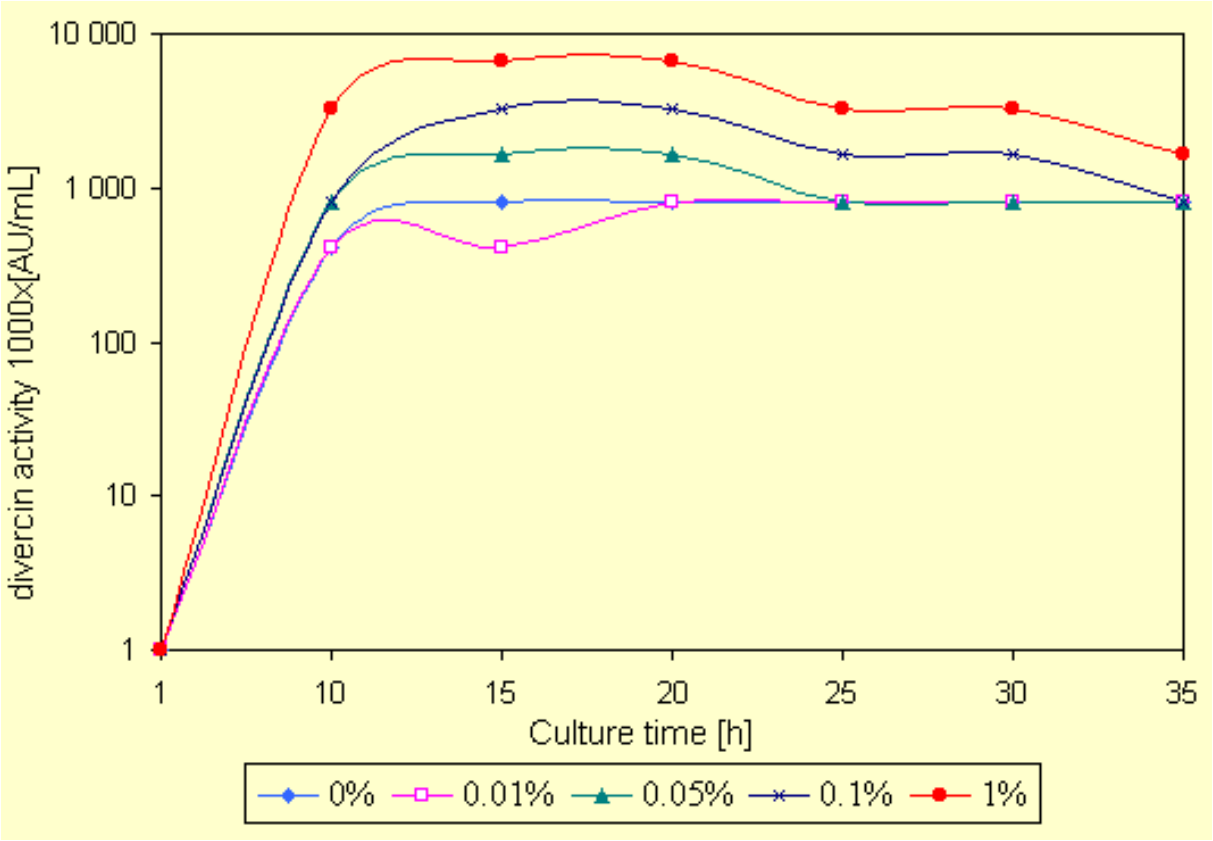
Ultrafiltration through the cellulose acetate membranes showed that in the presence of ethanol, isopropanol, Nonidet P-40 and Tween 80, used in their highest concentration, the divercin occurred in permeates mainly in the form of 10-30 kDa aggregates what is equivalent of polymers composed of 3-7 divercin molecules, whereas, in presence of SDS a dominant form of divercin were polymers obtained from membranes with cut of between 5-10 kDa, thus, composed of dimers.

The use of detergents or organic solvents can be proposed as a solution to disaggregate divercin polymers occurred in post-cultural fluids and can be considered as a step of down-stream processing in view to produce purified divercin preparation. However, the continuous separation of bacteriocin from culture media during continuous fermentation process with cell recycling in membrane bioreactor is also an important problem. In this application a detrimental effect of detergents and solvents on the bacteria viability can be expected. So, in this investigation a series of experiments were conducted in which detergents and solvents

were introduced into culture medium during bacteria cultivation. The disaggregation of divercin polymers as well as bacteria growth were taken simultaneously into consideration.

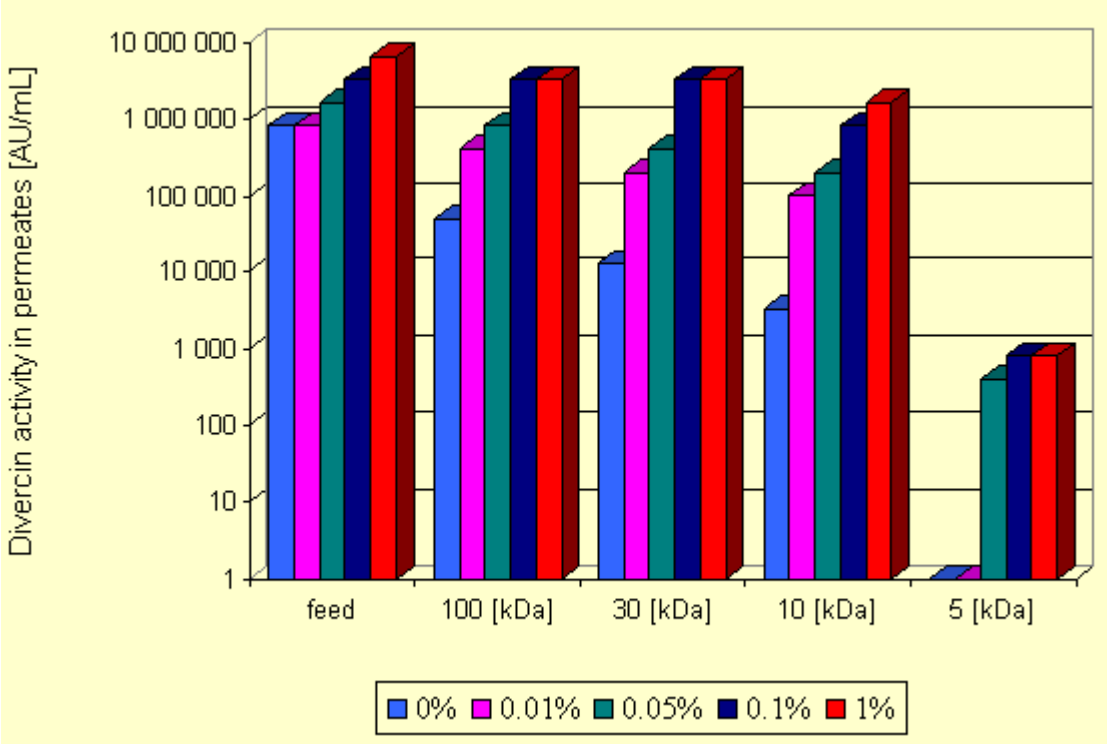
It was found that bacterial growth was continued only in the medium with Tween 80 addition. Other detergents and alcohols introduced into culture medium demonstrated a toxic effect to *Carnobacterium divergens* bacteria and totally stopped the fermentation process. A partial lysis of bacteria cells was also observed. Thus, in the fermentation carried out in the medium with 1% of Tween 80 addition the divercin activity was eight times higher then in the control (Fig. 3). It is also evident that positive influence of Tween 80 addition on divercin activity was achieved at the detergent concentration over 0.01% (w/v). The inhibition of cell growth by some detergents was observed with *Lactobacillus delbrueckii* subsp. *lactis* [Muriana and Klaenhammer 1991], *Carnobacterium piscicola* [6] and *Lactobacillus delbrueckii* subsp. *bulgaricus* [2].

Fig. 3. Effect of the presence of Tween 80 in culture medium on the production of divercin



Very interesting results were obtained in the experiments with ultrafiltration of post-culture media containing Tween 80 through cascade of cellulose acetate membranes (Fig. 4). It was found that the addition of Tween 80 to the *C. divergens* culture significantly improved divercin passage through membranes within whole range of Tween 80 concentrations. The satisfactory effect of this detergent on the divercin passage through the membrane cascade at the concentration of 0.1% (w/v) was achieved. Taking into account total effect of Tween 80 on the improvement of divercin production and divercin transfer through membranes the concentration mentioned above seems to be optimal.

Fig. 4. Divercin activity in permeates obtained from *C. divergens* AS7 culture carried out with Tween 80 addition by ultrafiltration of active extracts through cellulose acetate membranes



It should be also noticed that a part of divercin activity – about 400-800 AU/mL – passed through 5 kDa membrane and this result was achieved with the of Tween 80 concentration over 0.05 %. This effect was not observed in the case when detergent was introduced into supernatants of post-culture fluids (active extracts) after finishing of fermentation process (Fig. 2).

Using active extracts from the culture supplemented with Tween 80 , the highest divercin retention was detected on the surface of 5 kDa membrane, whereas, in the case of bacteria cultivation without Tween 80 addition and its introduction into post-cultural fluids the maximum of bacteriocin retention was observed on the 100 kDa membrane (Fig. 2 and 4). These facts allows to conclude that direct introduction of Tween 80 into culture medium during bacteria growth is the most efficient in the bacteriocin disaggregation.

ACKNOWLEDGEMENTS

Dr. Anna Sip is the holder of scholarship of the Foundation for the Polish Science 2001.

REFERENCES

1. Delves-Broughton J. ,1990, Nisin and its application as a food preservative. J. Soc. Dairy Technol. 43: 73-76.
2. Fitzgerald G.F., Vaughan E.V., Daly C., 1992, Identification and characterization of helveticin V-1829, a bacteriocin produced by Lactobacillus helveticus 1829. J. Appl. Bacteriol. 73: 299-308.
3. Hansen J.N., 1994, Nisin as a model food preservative. Crit. Rev. Food Sci. Nutr. 34: 69-93.
4. Joerger M. C., Klaenhammer T. R., 1986, Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481, J. Bacteriol. 167: 439-446.
5. Kabuki T., Saito T., Kawai Y., Uemura J., Itoh T., 1997, Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6. Int. J. Food Microbiol. 34: 145-156.
6. Khouiti Z., Simon J.P., 1997, Detection and partial characterization of bacteriocin produced by Carnobacterium piscicola 213. J. Ind. Microbiol. Biotechnol. 19: 28-33.
7. Lyon W. J., Glatz B. A., 1993: Isolation and purification of propionicyn PLG-1, a bacteriocin produced by a strain of *Propionibacter thoenii*, Appl. Environ. Microbiol. 59: 83-88.
8. Metivier A., Pilet M. A., Dousset X., Sorokine O., Anglade P., Zagorec M., Piard J. Ch., Marion D., Cenatiempo Y., Fremaux Ch., 1998, Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. Microbiology 144: 2837-2844.
9. Mortvedt C. J., Nissen-Mayer J., Sletten K., Nes I. F., 1991, Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl. Environ. Microbiol. 57: 114-121.
10. Muriana P. M., Klaenhammer T. R., 1991, Purification and partial characterization of lactocin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57: 114-121.
11. Pilet M. F., 1994, Characterisation de la divercine V41 et de la piscicocine V1, bacteriocines produites par *Carnobacterium divergens* V 41 et *Carnobacterium piscicola* V1 isolees de produits marins. Ph. Thesis, Universite de Caen, Institut de Recherches en Biologie Appliquee, pp 1-139.
12. Pilet M. F., Dusset X., Barre R., Novel G., Desmazaeud M., Piard J. Ch., 1995, Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes.* J. Food Protect. 58: 256-262.
13. Schved F., Lalazar A. Henis Y., Juven B. J., 1993, Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 74: 67-77.
14. Sip A., Grajek W., 1997, Production of divercin by *Carnobacterium divergens* in presence of divercin-sensitive bacteria *Carnobacterium piscicola* and *Listeria innocua*. Pol. J. Food Nutr. Sci. 3: 1-6.

Submited:

Anna Sip, Włodzimierz Grajek
Department of Biotechnology and Food Microbiology
Agricultural University of Poznań
Wojska Polskiego 48, Poland, 60-627 Poznań
tel. (+4861) 846 60 02
e-mail: aniasip@owl.au.poznan.pl, grajek@owl.au.poznan.pl,