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BIOSYNTHESIS OF DIVERCIN BY *CARNOBACTERIUM DIVERGENS* AS7 IN CONTINUOUS HIGH CELL DENSITY CULTURES

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ABSTRACT

Continuous cultures of *Carnobacterium divergens* AS7 with cell recycling were characterized with respect to their growth kinetics, fermentative activity and ability to biosynthesize the anti-listerial bacteriocin divercin. The experimental variables were dilution rates from 0.14 h^{-1} to 0.36 h^{-1} and a two-level recycling rate. The experiments demonstrated that dilution rate and cell recycling strongly influenced cell density in a bioreactor. With an increase in dilution rate, an increase in cell density was also observed. At D=0.36 h^{-1} the maximum cell density of 48 g d.m. l^{-1} was obtained. With long fermentation time and high cell density, the number of dead cells rapidly increased. In these experiments, when cell density increased 10-fold, the number of viable cells increased only 2-fold. It was found that divercin biosynthesis was reciprocally correlated to dilution rate. The greater dilution rate, the lower the volumetric productivity of the bioreactor became. The maximum volumetric productivity was obtained at D=0.14 h^{-1} and reached 8.6×10^8 AU $l^{-1}h^{-1}$. At high dilution rates and high cell densities, most of the divercin was cell bound. To keep appropriate permeate flux through the microfilter membrane, increases in transmembrane pressure and recycling rate were indispensable. The increase in recycling rate resulted in an increase in divercin activity in the permeate. Lactic acid production by *Carnobacterium divergens* was very low and did not exceed 1.5 g l^{-1} when the initial glucose concentration in the culture medium was 20 g l^{-1} and sugar utilization reached about 99.9%. The results obtained in this study demonstrate that continuous fermentation with cell recycling is an effective way to produce divercin.

Key words: Divercin, *Carnobacterium divergens*, membrane bioreactor, high cell density culture

INTRODUCTION

Recently, there have been many reports on microorganisms producing bacteriocins as well as on the chemical structure and biological activities of these substances [12]. However, it should be noted that information on the production of the bacteriocins in continuous fermentations is very scarce. The data reported refer mostly to batch cultures carried out in small volume vessels, usually in Erlenmeyer flasks [8, 9, 13, 19, 21, 23, 24, 26, 28]. To date, there have been few descriptions of experiments performed on the laboratory bioreactor scale [8, 15, 19, 21.23]. Efforts have also been made to increase bacteriocin yield by using immobilized cells [7, 27, 29] or using a membrane bioreactor [26].

One of the most interesting bacteriocins produced by the bacterium *Carnobacterium divergens* is divercin. It is a thermostable, proteinaceous substance with a molecular weight of about 4.3 kDa. It is resistant to heating at 100°C for 30 min and at 121°C for 10 min and is inactivated only when autoclaved for 15 min. Divercin is also stable in the presence of SDS, Tween 80 and urea. This bacteriocin shows bactericidal activity against *Listeria monocytogenes, Listeria innocua, Enterococcus faecalis*, some strains of *Carnobacterium piscicola,* and *Clostridium tyrobutyricum.* It demonstrates no antibacterial activity against lactic acid bacteria belonging to genera *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc* or against Gramm-negative bacteria [18, 24]. These properties make divercin a potential preservative for the fish, meat and dairy products.

The aim of the present study was to investigate the high cell density culture of *Carnobacterium divergens* AS7 performed in a membrane bioreactor in respect to the growth and production of divercin.

MATERIALS AND METHODS

Microorganisms

Divercin was produced by the *Carnobacterium divergens* AS7 strain, previously isolated from the salmon digestive tract. The bacteria were grown in anaerobic conditions at 30°C in a modified MRS medium which was sterilized at 121°C for 60 min [22, 25]. The *Carnobacterium piscicola* NCDO 2765 strain (E.N.I.T.I.A.A. Nantes, France) was used as an indicator organism to determine the divercin activity. These bacteria were cultured at temperature 30°C in a liquid medium containing 1% glucose, 1% NaCl and 0.5% yeast extract. Both microorganisms were stored in liquid nitrogen as a cell suspension in 50% v/v glycerol.

Continuous culture with cell recycling

Continuous fermentations with cell recycling were carried out in a membrane bioreactor system composed of a 5 dm^3 Bioflo III bioreactor (New Brunswick Sci., USA), peristaltic pump for medium recycling and microfiltration module with 0.22 μm CVDF membrane (model Prostak, Millipore, USA). The fermentations were carried out anaerobically in modified MRS medium at 30°C with a stirrer speed of 80 rpm. During cultivation, the pH value was maintained at 6.5 with 5 M NaOH. A 5.5 dm^3 of culture medium was sterilized at 121°C for 60 min. together with the bioreactor vessel, then cooled and inoculated with a 2% 14-hour-old inoculum of *C. divergens*. Each continuous fermentation was preceded by a 14 h batch fermentation. After this time, 1.5 dm^3 of fresh medium was added and medium recycling was initiated at a rate of 60 l h^{-1}. After 8 h of medium recycling in the bioreactor-microfilter loop, the continuous culture was started at a dilution rate (D) of 0.14 h^{-1}. The dilution rate was calculated on the basis of the total volume of the recycling system, including the working volume of the bioreactor, the circulation loop and the microfiltration module. Each dilution rate was maintained until a steady state was achieved. At this point, the dilution rate was increased to an higher level: at 54 h of culturing, D increased to 0.22 h^{-1} and was kept at this level for the next 24 h, afterwards, D increased to 0.28 h^{-1} for 16 h and the final period of fermentation was performed at D = 0.36 h^{-1}. A recycling rate of 60 l h^{-1} was maintained for 38 h and then increased to 120 l h^{-1}. During fermentation, the divercin activity (P_d), cell density (X), glucose concentration (S_b), lactic acid concentration (LA), and cell ATP in triplicate were determined.

Determination of the antibacterial activity of divercin

The divercin activity against an indicator bacteria *Carnobacterium piscicola* NCDO 2762 was determined in a culture supernatant collected from a bioreactor, culture permeate, and cell mass. The technique of critical dilutions described by Pilet et al. [19] for the assays was used. The divercin activity present in the analyzed samples was calculated in conventional units (AU ml^{-1}) which express the inverse of the lowest dilution exhibiting no activity against the indicator bacteria.

Dry matter of cell biomass

Cell biomass was harvested by centrifugation (8,000 g, 10 min), washed twice with a 0.01 M phosphate buffer of pH 6.5 (Calbiochem) and dried at 60°C for 5 h and again at 105°C for constant weight.

Viable cell count

The number of viable cells (X_v) was determined by the plate method and expressed as colony forming units (CFU) using the MRS agar medium.

Determination of ATP

The ATP contents in cell biomass were determined by the bioluminescence method using a luminometer (Lumac type Biocounter M 1500). The assay was performed using a Microbial Biomass Test Kit (Lumac, Netherlands), according to the procedure described by the producer. Luminescence was measured as relative luminescence units (RLU) and ATP was calculated using the formula: 1 RLU = 0.0277 pg ATP.

Determination of lactic acid and glucose

Lactic acid and glucose concentrations in the bioreactor were determined by HPLC using a Hewlett-Packard model HP 1050 equipped with a refractive-index detector (HP model 1047A), and a column Bio-Rad HPX-87H. The analyses were performed using 0.05 M sulfuric acid (Sigma) as effluent at a flow rate of 0.8 ml/min at ambient temperature. The samples were previously passed through a 0.22 μm Millipore filter and loaded onto a column at a volume of 50 μl.

Yield coefficients

The fermentations were characterized by the following yield coefficients:

- volumetric productivity of extracellular divercin; $Y_{Pd/V}$ [AU h^{-1}],
- specific productivity of extracellular divercin; $Y_{Pd/x}$ [AU $l^{-1}g^{-1}h^{-1}$],
- divercin yield from glucose unit; $Y_{Pd/S}$ [AU g^{-1}],
- utilization of glucose by *C. divergens* AS7; S_u [%].

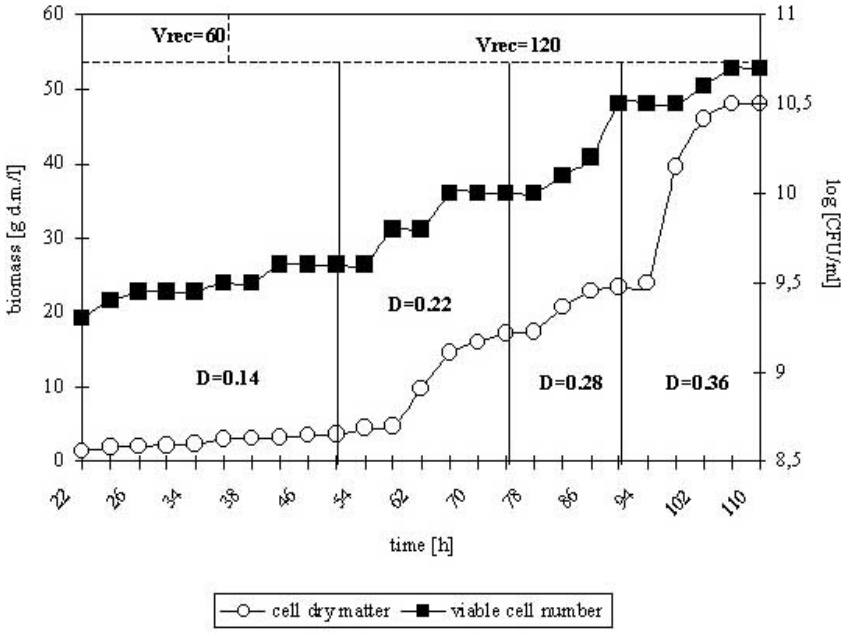
These calculations were made according to the formula defined by Sinclair and Cantero [20] and Brown [6].

RESULTS AND DISCUSSION

Bacteria growth and fermentation characteristics

The growth of bacteria depended on the cell recycling rate and the dilution rate. At D = 0.14 h^{-1} the cell density stabilized at 4.4 g d.m.l^{-1}. An increase of dilution rate caused an increase in cell density in the bioreactor (Fig. 1). At D = 0.36 h^{-1} cell biomass concentration reached a maximum of 48 g d.m. l^{-1}, which was equivalent to 7×10^{10} CFU ml^{-1}. This value was 60 times higher than the cell concentration obtained in a batch culture [10] and 16 times higher than in continuous culture without recycling [22]. Microscope analysis showed that bacteria tended to form aggregates with enlarged sizes when cell density increased.

Fig. 1. Growth kinetics of *C. divergens* AS7 in continuous culture at different dilution rates



The results indicate that the efficiency of the biomass production of *C. divergens* AS7 in continuous culture with cell recycling was comparable to other lactic acid bacteria cultivated in a membrane bioreactor at similar dilution rates [1, 11, 14]. Higher cell concentrations than those published in this work were achieved only at greater dilution rates, e.g. 80 g d.m. l^{-1} of *Lb. delbrueckii* was obtained at D=0.95 h^{-1} [16], whereas a cell density of *Lb. plantarum* was concentrated to 60 g d.m. l^{-1} at D=1.27 h^{-1} [2].

It was observed that the increase in cell density was accompanied by an increase in viable cell number (Fig. 2). However, the concentration of cell biomass grew faster than the concentration of viable cell number, which resulted in a decrease in the pecentage of viable cells in the bacterial population. The data presented in Table 1 shows that the number of viable cells calculated per gram of cell dry matter was 6-fold higher at D = 0.14 h^{-1} than that determined at D = 0.36 h^{-1}.

Fig. 2. Kinetics of lactic acid fermentation during continuous culture of *C. divergens* AS7 with cell recycling

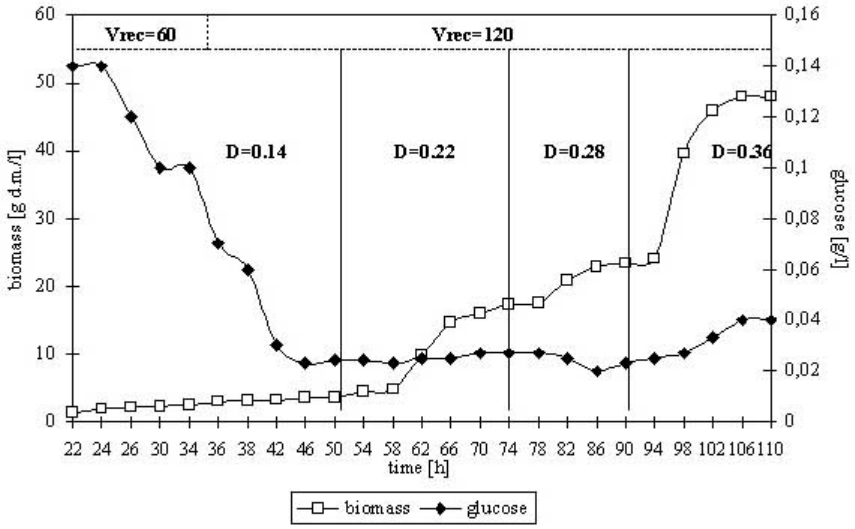


Table 1. Technological coefficients for continuous fermentation with cell recycling

D [h^{-1}]	S_b [gl^{-1}]	S_u [%]	LA [g/l]	X [g d.m. l^{-1}]	X_v [CFU ml^{-1}]	P_d 10^5[Au ml^{-1}]	$Y_{Pd/v}$ 10^8[AU $l^{-1}h^{-1}$]	$Y_{Pd/x}$ 10^7[AU g-1 h^{-1}]	$Y_{Pd/S}$ 10^7[AU g^{-1}]
0.14	0.024	99.9	1.15	4.4	3.0×10^{10}	8.192	8.60	18.61	4.10
0.22	0.027	99.9	0.70	17.5	5.0×10^{10}	4.096	6.76	2.34	2.05
0.28	0.025	99.9	0.80	24.0	5.0×10^{10}	1.024	2.15	0.43	0.51
0.36	0.040	99.8	0.80	48.0	6.0×10^{10}	0.512	1.38	0.11	0.26

P_d - extracellular activity of divercin in bioreactor

The decrease in cell concentration at a higher recycling rate was not caused by mechanical cell damage. It was demonstrated that the viable cell number was constant when the recycling rate increased from 60 l h^{-1} to 120 l h^{-1}. Microscopic analysis suggested cell autolysis as the cause of cell death. It was observed that bacteria changed their cell morphology and became shorter and oval with shorter fermentation time, which suggested a stress condition for their growth.

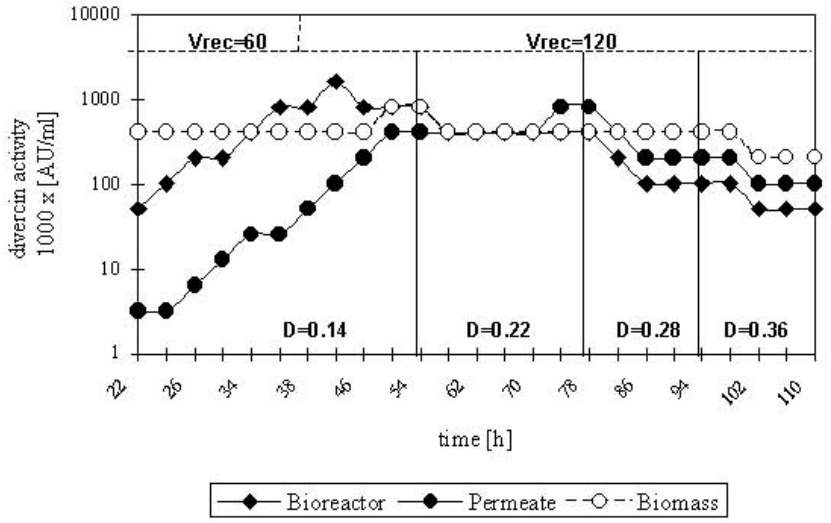
The data presented in Fig. 2 showed that glucose concentration rapidly decreased during fermentation. The initial glucose concentration was 20 g l^{-1} and after 46 h of fermentation, its concentration diminished to 0.024 g l^{-1}. This level was kept with small fluctuations up to 94 h and stabilized at 0.04 g l^{-1}. The glucose utilization reached over 99.8 % and glucose deficiency can be considered as a limiting factor in fermentation (Table 1). The maximum concentration of lactic acid reached 1.15 g l^{-1} at D= 0.14 h^{-1}. The increase in dilution rate resulted in a decrease in lactic acid concentration to 0.7-0.8 g l^{-1}. These data show that *Carnobacterium divergens* AS7 produced a small quantity of lactic acid and sugar was mostly used for cell biomass production and biosynthesis of other metabolites, e.g. bacteriocin. This feature of *C. divergens* can be considered as an advantage for the food industry because of limited product acidification.

The published data indicate that the fermentation activity of *C. divergens* AS7 was significantly lower than the activity of other LAB culturing in membrane bioreactors [1, 3, 4, 5, 11, 17, 30].

Divercin biosynthesis

At the beginning of continuous fermentation, the divercin activity in a liquid medium achieved only 51,200 AU ml^{-1} but when starting cell recycling, the divercin activity increased very rapidly and after 50 h of culturing had reached a level of 819,200 AU ml^{-1}. It was observed that at a low recycling rate divercin activity in the permeate was 8-16 times lower than in the bioreactor. The increase in recycling rate from 60 l h^{-1} to 120 l h^{-1} resulted in significant improvement of filtration efficiency and in the augmentation of divercin activity in the permeate (Fig. 3). After 74 hours, the divercin activity in the permeate reached a maximum of 1,638,400 AU ml^{-1} and had twice the extracellular activity of the bioreactor. The increase of divercin activity in the permeate may have been caused by the disaggregation of divercin macromolecules during the passage through the filtration membrane.
The increase in recycling rate also doubled the extracellular activity in the bioreactor. However, a temporary increase of divercin activity due to faster medium circulation was followed by a return to the previous activity level of 819,200 AU ml^{-1}.

Fig. 3. Dynamics of divercin biosynthesis in continuous culture with cell recycling



The dilution rate distinctly affected the extracellular activity of the divercin. The data presented in Fig. 3 show that divercin production was reciprocally related to the dilution rate. The highest level of divercin activity, namely 819,200 AU ml^{-1}, was detected at D = 0.14 h^{-1}. Unfortunately, this was followed by a significant decrease in divercin activity with further acceleration of the dilution rate. At D = 0.36 h^{-1} divercin activity was reduced to 51,200 AU ml^{-1}.

The dilution rate also influenced the volumetric productivity of the bioreactor. Generally, the volumetric productivity of divercin decreased with an increase in dilution rate. The peak value of this coefficient was obtained at D = 0.14 h^{-1} and amounted to 8.6×10^{8} AU $l^{-1}h^{-1}$. This was over six times higher than the productivity at D = 0.36 h^{-1}. The results of earlier investigations showed that it was four times higher than the maximum productivity of continuous fermentation without cell recycling [22, 25].

In comparing the volumetric productivity obtained in continuous culture [23, 24] and in continuous culture with cell recycling, the efficiency of the latter method is distinctly higher, especially when D ranged from 0.14 h^{-1} to 0.28 h^{-1}. The maximum productivity value in a culture with cell recycling was four times higher than in a classical continuous culture. An exception are the results obtained in the culture carried out at D=0.36 h^{-1}.
In the literature, few reports on the production of divercin in membrane bioreactor have been published. Taniguchi et al. [26] described the biosynthesis of nisin and declared that the volumetric productivity of this bacteriocin was $7.8\text{x}10^{4}$ AU $l^{-1}h^{-1}$ at D = 0.05 h^{-1}. This result is significantly less than that obtained with divercin in this work. The authors also found that the cell recycling in continuous culture increased nisin productivity by 4.5 times.

The data presented in Table 1 shows that the specific productivity of extracellular divercin in fermentation with cell recycling also depended on dilution rate. The maximum activity, namely 18.6×10^7AU per one gram of cell dry matter, was the greatest at D = 0.14 h^{-1} and decreased versus dilution rate increase. At D = 0.36 h^{-1} the specific productivity value of divercin was 175 times less than at D = 0.14 h^{-1}. Thus, the ability of a bacterial population to produce divercin diminished. In comparing earlier results related to continuous culture without cell recycling [22] to the data obtained in this study, cell recycling appears to have diminished the specific productivity of bacteria by about three times. This is probably due to the stress conditions in high cell density cultures. At high cell concentrations, the medium viscosity increased, which produced greater share forces and generated mechanical stress during cell recycling, which could have negatively affected bacterial metabolism.

During fermentation with cell recycling, cell biomass harvested from a bioreactor demonstrated a high antibacterial activity (Table 1). It fluctuated from 409,600 AU ml^{-1} to 819,200 AU ml^{-1} at dilution rates ranging from 0.14 h^{-1} to 0.28 h^{-1}. With an increase in cell concentration, most of the divercin was cell bound. At D = 0.36 h^{-1} the divercin activity bound to cell surface decreased to 204,800 AU ml^{-1}. At high cell densities, the activity of divercin bound to the cell surface was 4-fold higher than the extracellular activity. It means that at low dilution rates (0.14 h^{-1}-0.22 h^{-1}) about 40% of divercin activity was cell bound, whereas at high dilution rates (0.28-0.36 h^{-1}) over 80% of divercin was bound to the cell surface.

ATP accumulation

The data presented in Tables 1 and 2 showed that changes in the cell density were correlated with changes in ATP contents. With the augmentation of cell biomass concentration, the ATP amount per volume unit also increased. It is interesting that ATP content, calculated per gram of cell dry matter, decreased with an increase in dilution rate and cell concentration. The differences between ATP content in biomass collecting at D=0.14 h^{-1} and D=0.36 h^{-1} were very significant. It could have been due to the large number of dead cells in the total population. Despite this, the specific yield of ATP production increased from 0.003 fg/CFU at D=0.14 h^{-1} to 0.005 fg/CFU at D=0.36 h^{-1}. It can be explained by better nutrition with glucose at a high dilution rate. It should be also stressed that these values were 8 times lower than the ATP contents determined in bacteria cells cultivated in batch cultures (data unpublished). It suggests that the conditions in batch cultures are preferential for bacterial cells from an energetic point of view.

Table 2. ATP contents in cells of *C. divergens* AS7 separated from continuous cultures carried out at different dilution rates

D	ATP contents		
[h^{-1}]	[ng ml^{-1}]	[µ g g^{-1} d.m.]	[fg CFU^{-1}]
0.14	80	18.2	0.003
0.22	200	11.5	0.004
0.28	250	10.4	0.005
0.36	320	6.6	0.005

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