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A SUITABLE PROCEDURE TO CHOOSE ANTIMICROBIALS AS CONTROLLING AGENTS IN FERMENTATIONS PERFORMED BY BACTERIA

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SHORT COMMUNICATION

ABSTRACT

This work evaluated the influence of nitrofurantoin, erythromycin and streptomycin at 50, 25 and 12,5% of the minimal inhibitory concentration (MIC) on maximum specific growth rate (μ_{max}) and specific polymer accumulation rate (μ_{PHB}) of A*lcaligenes eutrophus*, considered resistant to those antimicrobials. Nitrofurantoin strongly affected μ_{max} even at 50% MIC. Streptomycin moderately affected μ_{max} only at 50%MIC. Nitrofurantoin showed the most harmful effect on μ_{PHB} when 50% MIC was applied and erythromycin was not harmful.

Key words: antimicrobial susceptibility, MIC, PHB, *Alcaligenes eutrophus, Ralstonia eutropha*

Procedures usually indicated to prevent contamination on fermentative processes include sterilization of equipment and medium, careful manipulation during inocula preparation, bacterial cultivation and product formation. Since sterilization and prevention of contamination in large scale processes are expensive, energy- and time-consuming, additional procedures would be useful to eliminate contaminants, such as the use of antimicrobials (2, 10). Choosing an antimicrobial with the desirable selective toxicity is difficult and the exact amount to be applied must be previously determined since an excessive concentration may modify the global performance of the microorganism of interest or remain in the product altering its properties.

Though many reports can be found on antibiotic susceptibility of *Alcaligenes* species of medical interest, there are not references about *A. eutrophus* (1) used in the production of polyhydroxybutyric acid (P3HB). P3HB and its copolymer poly-(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (P3HB-*co*-3HV) have attracted great industrial attention as biodegradable plastics (8). They are generally produced in two steps: the first one aims at reaching high density of *A. eutrophus* cells and the second one at accumulation of large amounts of P3HB (4). Bacterial contamination may occur in both steps as the medium usually does not contain antimicrobial agents and large batches of fermentation may therefore be lost (5, 9).

In this work three antimicrobial agents were tested at sub-MIC concentrations regarding to their effects on kinetics of growth and P3HB accumulation by *A. eutrophus*.

A. eutrophus DSM 545 (recently reclassified as *Ralstonia eutropha*) was submitted to disk diffusion test (3) at 30ºC and minimal inhibitory concentration (MIC) using the microdilution test (6). MIC was determined for three antimicrobials to which *A. eutrophus* was resistant in standard disk diffusion test (data not shown). MICs obtained were 8, 125 and 250 µg/ml for erythromycin, streptomycin and nitrofurantoin, respectively. Since MIC is based on growth or non-growth results, without any growth or accumulation kinetics data provided, experiments were performed to evaluate to what extent sub-MIC

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concentrations would still modify the maximum specific growth rate (μ_{max}) and also the specific P3HB accumulation rate (μ_{PHB}). In growth experiments, glucose plus fructose (5g.l^{-1} each) and $(NH_4)_2SO_4$ (3 g.l^{-1}) were supplied in mineral salts medium containing two fold original concentration of phosphates and 1.5 fold $MgSO_4$ (11). In accumulation experiments, a nitrogen free mineral salts medium containing glucose (10 g.l^{-1}) was tested. The antibiotics were added in concentrations 2, 4 and 8 fold lower than the MICs. In growth experiments, samples were taken at every 2h to analyse: optical density at 610 nm (OD_{610}) (Spectronic Genesys 5 spectrophotometer) and pH (Mettler Delta 350 pHmeter). Values of μ_{max} were calculated choosing by inspection the best points from the plot $LnOD_{610}$ versus time and calculating the slope of the straight line using the minimum squares method as indicated in Fig. 1. In accumulation experiments, samples were taken at every 4 h to evaluate cellular dry biomass (7) and P3HB concentration (7). Values of μ_{PHB} were obtained from the plot: ratio of P3HB concentration by the average of residual biomass (X_r, i.e, non-P3HB biomass) versus time.

Reference μ_{max} of *A. eutrophus* on mineral medium with glucose plus fructose was 0.30 ± 0.01 h^{-1} (average and standard deviation of 4 repetitions). *A. eutrophus* was resistant to nitrofurantoin in disk diffusion test but MIC results indicated that the growth would be inhibited at an approximate concentration of 250 µg/ml. However, when submitted to 125 µg/ml, μ_{max} decreased from 0.30 to 0.09 h^{-1}, indicating that nitrofurantoin strongly affected μ_{max} even at 50% of MIC. Even concentrations amounting 25 and 12.5% of MIC affected considerably μ_{max} which reached 0.24 and 0.26 h^{-1}, respectively. A reduction on antibiotic concentration from 125 to 31 µg/ml did not eliminate a prolonged lag phase (Fig.1), despite μ_{max} have raised from 0.09 to 0.26 h^{-1}. On the other hand, erythromycin did not affect bacterial μ_{max} in the three concentrations tested since values obtained were between 0.29-0.30 h^{-1} (plots not shown). Streptomycin was at an intermediate position and exerted a moderate effect on μ_{max} in all sub-MIC concentrations tested. Although in presence of 50% MIC, μ_{max} decreased to 0.25 h^{-1}, this value was 0.29 h^{-1} when the other two concentrations were tested.

Results obtained indicate that a contaminant arising during growth phase should be tested against antibiotics at concentrations that maintained μ_{max} of *A. eutrophus* between 0.29 - 0.31 h^{-1}. Thus a sensitive contaminant could be eliminated without interfering in the growth of the main bacteria. The use of nitrofurantoin might be possible at 12.5% of MIC if no other alternative is available as it strongly affects the standard strain during growth phase. Therefore the application of conditions capable to keep *A. eutrophus* at a $\mu_{max} \geq 0.29$h^{-1} are recommended in the control of sensitive contaminant bacteria.

The reference μ_{PHB} for *A. eutrophus* in nitrogen free mineral medium, with 10 g/l^{-1} of glucose without antibiotic (average of 4 repetitions) was 0.09 ± 0.02 g_{PHB}/g_{cells}.h. Nitrofurantoin did not modify μ_{PHB} only when 12.5% of MIC was used (μ_{PHB} = 0.08 g_{PHB}/g_{cells}.h), being harmful to bacterial P3HB accumulation at higher concentrations. At 25% MIC, μ_{PHB} was 0.05 g_{PHB}/g_{cells}.h.

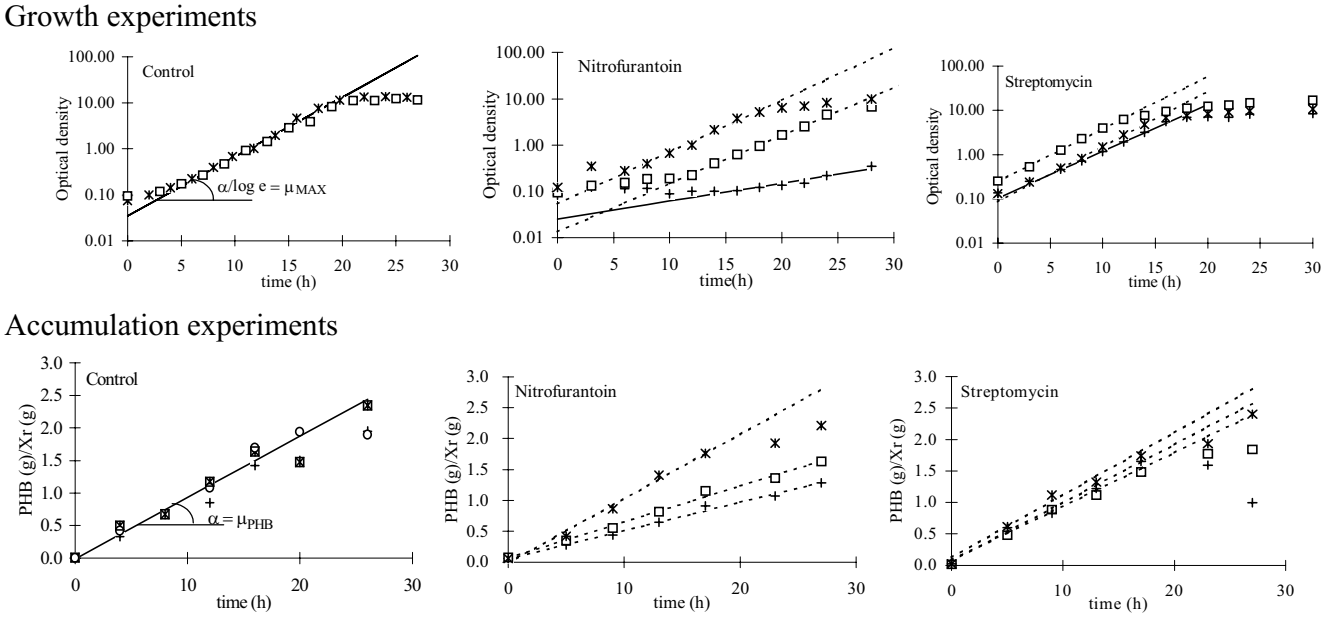

Figure 1. Results and plots used to calculate μ_{max} and μ_{PHB} of *A. eutrophus* without and with antimicrobials selected: erythromycin, streptomycin and nitrofurantoin at concentrations corresponding to (+) 50% MIC, (□) 25% MIC and (*) 12.5% MIC.

The most harmful effect was observed when 50% of MIC was applied reducing μ_{PHB} to 0.04 g_{PHB}/g_{cells}.h. Erythromycin and streptomycin did not affect bacterial μ_{PHB}, since values obtained were between 0.08 and 0.11 g_{PHB}/g_{cells}.h. Nistatin effect was tested in similar experiments applying double and half of the concentration used to inhibit yeast contamination (60 $\mu g.ml^{-1}$) and did not affect μ_{PHB} neither μ_{max}.

The successful control of bacterial contamination by antimicrobials in bacterial fermentative processes should take into account the susceptibility of both the contaminant and the microorganism responsible for the process. Since there are usually only slight differences between both organisms concerning to structure, morphology and physiology, the choice of antimicrobials for contamination control based on selective toxicity is difficult. This choice must first consider the susceptibility of the industrial microorganism.

In this work, a procedure is proposed to identify the antibacterials from different chemical groups plus the antimycotic nistatin that can be applied in the control of microbial contamination during P3HB production by *A. eutrophus*. Once determined those antimicrobials to which the industrial microorganism is resistant, they should be tested against a contaminant detected in the fermentation process. In the case studied, amongst 3 antibacterials to which *A. eutrophus* was resistant, nitrofurantoin should be used carefully against contaminant since it expressively modifies bacterial μ_{max} and μ_{PHB} even at sub-MIC concentrations. Erythromycin and streptomycin did not affect μ_{PHB} and could control a sensitive contaminant detected in the accumulation phase. It is important to remind that a decreasing in the value of μ_{max} from 0.30 to 0.26 or 0.09 h^{-1} would represent an extension on the time required for the industrial fermentation process of about 15 and 70 %, respectively and could result in significant economical loss.

The disk diffusion test and MIC are useful tools to be applied as preliminary susceptibility determinations to select antimicrobials to control contamination in fermentative processes. Although MIC values can be used as reference, specific tests must be performed to evaluate the real influence of antimicrobials on the industrial strain under production situations to assure a safety handling of contamination if it occurs, avoiding economical loss.

This approach could be applied to processes of any fermentative product. In addition to the routine procedures to prevent contamination, a careful selection of the antimicrobial and concentrations that can be applied should be accomplished. A criterious evaluation about the scale application, environmental impacts and even the influence in the product obtained must be also taken into account.

RESUMO

Procedimento para escolha de antimicrobianos como agentes de controle em fermentações por bactérias

Avaliou-se o efeito de concentrações inferiores à mínima inibitória (50, 25 e 12,5% do MIC) de nitrofurantoína, estreptomicina e eritromicina sobre velocidade específica máxima de crescimento (μ_{max}) e velocidade específica de acúmulo de polímero (μ_{PHB}) em *Alcaligenes eutrophus*, considerada resistente a estes antimicrobianos. Nitrofurantoína modificou bastante μ_{max}, mesmo com 50% MIC. Estreptomicina afetou moderadamente e eritromicina não afetou μ_{max}. Quanto à produção de polímero, nitrofurantoína alterou fortemente μ_{PHB} quando se empregou 50% MIC.

Palavras-chave: sensibilidade a antimicrobianos, MIC, PHB, *Alcaligenes eutrophus*, *Ralstonia eutropha*

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