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TECHNICAL NOTE

The influence of centrifugation on *Zymomonas mobilis* aggregation

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Flocculent *Zymomonas mobilis* breaks down in smaller flocs and individual cells when centrifuged. The main consequence of it is an increase in the dispersion of the sample, suggesting that the influence of the centrifugal force on the aggregation of cells is worth to study. The experiments showed that the degree of dispersion varied between 30% and 100% when the centrifugal acceleration changed from 25 g to 2500 g. Observation under the electronic microscope showed that a slimy material covered the cells recovered by gentle gravitational settling and, that the centrifuged cells presented a bare cell wall.

Zymomonas mobilis has attracted interest as an agent for ethanol production asit presents a better kinetic performance than *Saccharomyces cerevisiae* (Rogers et al. 1982; Toran-Diaz et al. 1983; Borrego et al. 1987; Kademi and Baratti, 1996). However, recent interest has been directed towards the industrial production of gluconic acid and sorbitol, because of the much higher added value. Different industrial uses of *Zymomonas mobilis* have been proposed. Among them, the production of levan (Calazans et al. 1997; Vigants et al. 1998), the fermentation of hydrolysed cellulose (Kademi and Baratti, 1996) and the fermentation of glucose in high acetate concentration (Joachimstal et al. 1998) are worth to mention.

Microbial flocculation facilitates the separation of the cells by increasing particle size. This phenomenon allows the use of gravitational thickeners for cell separation. Cellular aggregates have been extensively used for fermentation in brewery (Hamersveld et al. 1997) and wastewater treatment

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(Bossier and Verstraete, 1996). Many studies have been carried out about yeast flocculation, mostly with *Saccharomyces cerevisiae,* to reveal the aggregation mechanism. This mechanism considers hydrogen bonds, lectin like substances, calcium and manganese salts and polysaccharides as responsible for flocculation (Stratford, 1992; Pereira Jr. and Bu'lock, 1993). The effect of growth kinetics and the nature of the polyelectrolytic flocculants on cell flocculation have been studied in *Zymomonas mobilis* (Rogers et al. 1982; Hughes et al. 1994). The presence of a glucosidic or a fructosidic capsule has been claimed as responsible for the aggregative properties of this bacterium (Barrow et al. 1984; Kirk et al. 1994).

The main industrial application of cell aggregation occurs in the brewing industry, where the selection of the yeast strain is based on its flocculating behaviour, which determines the type of beer to be produced and the fermentation conditions. Flocculating yeasts produce beers with better aspect, clearer appearance and better taste (Rose, 1984; Martinez, 1998). Other traditional use of flocculation is in wastewater treatment (Bossier and Verstraete, 1996; Hamersveld et al. 1997).

The physical characteristics of *Zymomonas mobilis*flocs are particularly suitable for industrial use. However, very few works have been done to elucidate the mechanisms that lead to flocculation (Lopes et al. 1991; López et al. 1996; Palha et al. 1997). On the other hand, the flocs of *Zymomonas mobilis* separated from the fermented broth by centrifugation are usually dispersed. Based on this information, the effect of the centrifugal acceleration on the aggregation of *Zymomonas mobilis* strain CP4 was studied.

Materials and Methods

Microorganism and culture conditions

Zymomonas mobilis strain CP4 (DAUFPE 202) was obtained from the culture collection of the Antibiotics Department of the Universidade Federal de Pernambuco, Brazil. The culture was stored refrigerated at 4ºC in SSDL medium containing (g/L): glucose, 20.0 and yeast extract, 5.0. The composition of the medium used in fermentation and for the preparation of inocula was (g/L): glucose, 100.0; yeast extract, 2.0; urea, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5 and was sterilized at 120ºC for 20 minutes. The cells were grown in flasks at 30ºC and initial pH of 6.5 without control.

Evaluation of flocculation

The dispersion degree was expressed in terms of the percentage of cells that remained in suspension (R) as proposed by Pereira Jr. and Bu'lock, 1993:

$$R = \frac{C_L}{C_T} * 100$$

where C_T and C_L represent the concentrations of total and free cells in suspension, respectively. Both were determined as dry weight and R given as percentage.

Effect of centrifugation on aggregation

The cells were collected at the end of stationary phase by settling at 18 hours of fermentation and washed three times by resuspending them in 100 mL of distilled water to remove the remaining sugar. Ten millilitre samples of the suspension at neutral pHwere transferred to measuring centrifugation tubes and submitted to gentle agitation during one minute. The decrease in the height of the settling zone was monitored to determine the time in which the volume of the settled zone reached 3 mL. This time averaged 2':30" + 10". Then, each pair of tubes was submitted to 25 g, 100 g, 400 g, 900 g, 1600 g and 2500 g, for 15 min. The centrifuged cells were dispersed and the suspension was settled for 35 min.Samples were then collected to determine the free cells in suspension and total cells to calculate the parameter R. The time of 35 min. was chosen for being the time required to obtain a 3 mL-settled zone when the cells were centrifuged at 1600 g. Centrifugation was carried out using an Excelsa I centrifuge model 206 with a radius of 9 cm.

Analyses of the CP-4 strain by the electronic microscopy

Two samples were analysed. One of them was centrifuged at 2500g for 15 minutes and the other was settled by gravity. Both samples were prepared for the scanning electronic microscope according to the procedure described by Bozzola and Russel, 1992.

Results and Discussion

Results of flocculation at different centrifugal forces, are presented in Figure 1. R increased considerably, from 29% at 25 g to 65% at 400 g. It remained almost constant until 1700 g and then, it increased sharply to R =100% at 2500 g when the cells were totally dispersed. No conclusive explanation for this phenomenon has been found in the literature; however it may be caused to the loss of the glycocalyx, as suggested by Barrow et al. 1984.

The behaviour of *Zymomonas mobilis* strain CP-4, when submitted to centrifugation, was studied using scanning electron microscopy. Figure 2 illustrates an electronic micrograph of a cellular aggregate not subjected to centrifugation. It can be seen that the floc is highly compact and composed by a very high number of cells.

The same floc is shown in Figure 3 at a higher magnification (5200X). Now a slimy material can be seen covering the cells. This material could be the glycocalix, as described in the literature, found in many microorganisms such as *Xanthomonas* (Bozzola and Russel, 1992). This exocellular substance seems to have a decisive role in cellular aggregation.

Electronic micrographs of cells from the flocculating strain CP-4 obtained after centrifugation at 2500 g are shown in Figure 4 and Figure 5. In this case, dispersed cells are observed, devoid of the exocellular slime and bearing broken *pili* .This suggests that this slime material cemented the cells in the floc, and that the loss of this material is responsible for the loss of flocculence after centrifugation.

Concluding Remarks

The aggregation of *Zymomonas mobilis* strain CP-4 was severely impaired as a consequence of centrifugal forces, with a dispersion degree increasing from 30% at 25 g to 100% when submitted to centrifugal acceleration of 2500 g or higher. The flocculating cells, when subjected to scanning electronic microscopy, revealed to be covered by a slimy substance. that, besides others roles, seems to contribute to the formation of the flocs. The scanning electron microscopy of flocculating cells of strain CP-4, that have been submitted to centrifugation at 2500 g or higher, showed the dispersed condition of the cells. Under those conditions, the exocellular material has been removed. In this way, one can conclude that high centrifugation forces, produce the total or partial removal of the biocap of this Gram-negative bacterium, which seems to be closely related to cell aggregation.

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APPENDIX

Figures

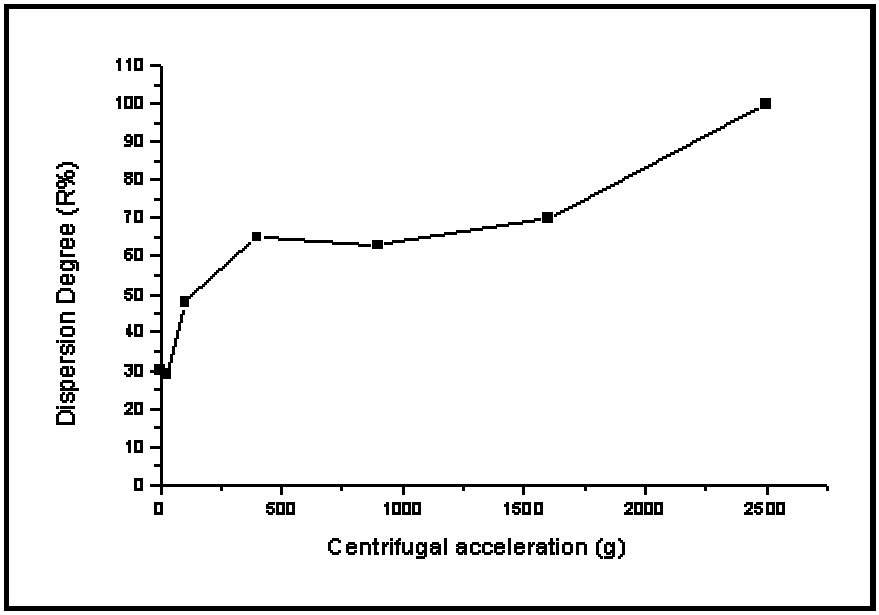

Figure 1. The effect of centrifugal acceleration on the degree of cell dispersion at 35 minutes of settling time.

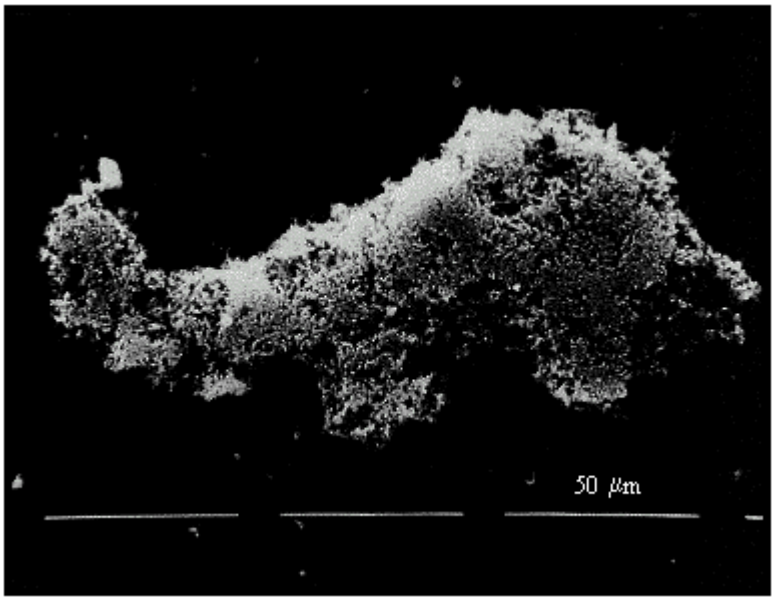

Figure 2. Flocs of *Zymomonas mobilis* strain CP-4 sampled directly from the fermented broth (540X).

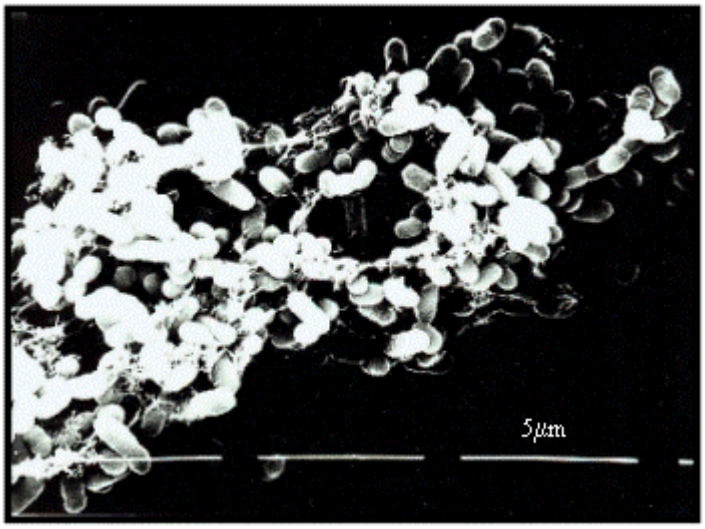

Figure 3. Flocs of *Zymomonas mobilis* strain CP-4 sampled directly from the fermented broth (5200X).

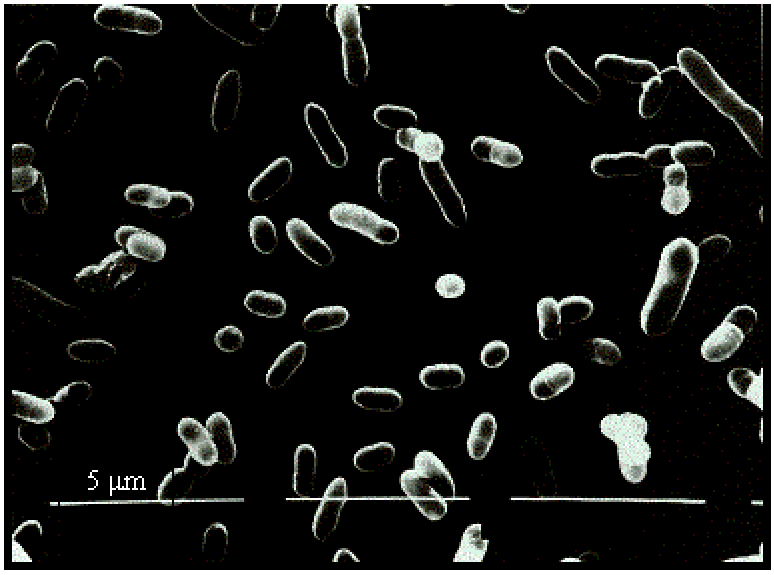

Figure 4. Resuspended cells of *Zymomonas mobilis* strain CP-4 after centrifugation at 2500 g (5600X).

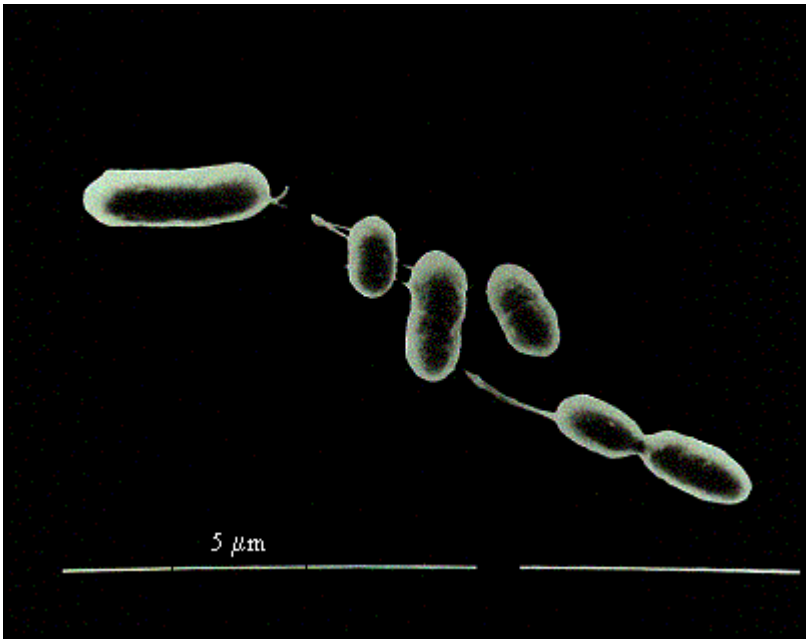

Figure 5. Fragments of cell flocs whose *pili* appear disrupted by centrifugation (10.800X).