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Immobilized cells: behaviour of carrageenan entrapped yeast during continuous ethanol fermentation

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Summary. Data of cell concentration, viability and microscopic observation of cell distribution inside carrageenan immobilized yeast beads are reported. Results were obtained from a continuous packed-bed reactor performing alcoholic fermentation and the main observations made on cell activity are in agreement with the fermentation profiles inside the fermenter.

Introduction

Immobilized cell systems have been the subject of intensive research during the past fifteen years, and they have been applied to the production of many compounds (Chibata et al. 1983; Kennedy and Cabral 1983; Linko and Linko 1984). Ethanol fermentation in particular has been studied by many authors; a review of the literature is to be found in Margaritis and Merchant (1984). The most widely used immobilization methods are those based on cell entrapment in gels of natural origin, like carrageenan and Ca-alginate. Although the performance of various types of continuous fermentation systems using these methods has been reported by several authors (Cho et al. 1981; Chotani and Constantinides 1984; Luong 1985; Nagashima et al. 1984; Wada et al. 1981; Williams and Munnecke 1981), less attention has been paid to cell evolution in the fermenters and inside the gel beads (Furusaki et al. 1983; Siess and Divies 1981; Wada et al. 1980; Wang and Hettwer 1982). The aim of this work is to contribute to the knowledge about this aspect of immobilized cells.

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Materials and methods

Microorganisms. A yeast strain isolated from high grade wine and identified as *Saccharomyces cerevisiae* Hansen was used.

Immobilization. Yeast cells were immobilized by entrapment in carrageenan gel beads (Genugel X-0828 type, kindly donated by the Copenhagen Pectin Co. Ltd., Lille Skensved, Denmark). The cell suspension to immobilize was added to a previously sterilized carrageenan solution and the mixture was pumped through a 21-gauge hypodermic needle into a cold, gently stirred 2% $CaCl_2$ solution. Spherical beads of 3.5 mm diameter were obtained. After 30 min, the $CaCl_2$ solution was removed and a 2% KCl solution was added. The immobilized cell beads were incubated in feed medium with 100 g l^{-1} of glucose for 1—2 day in order to increase their cell concentration prior to using them in the continuous fermentation experiments.

Continuous fermentation. Continuous fermentation was carried out in a vertical packed-bed reactor, 40 cm long by 2.54 cm in diameter, with four samle ports at 5, 15, 25 and 35 cm from the bottom. The medium was fed at the bottom of the fermenter, and had the following composition (per liter): glucose, 50—200 g; yeast extract, 2 g; NH_4Cl, 1.3 g; $MgSO_4$, 0.82 g; KH_2PO_4, 2 g; sodium citrate, 1.1 g and citric acid, 1.5 g. Various continuous runs were made under the following experimental conditions: temperature was 25° C, 30° C or 35° C; glucose concentration in the feed medium in g l^{-1} was 71, 95, 150 or 185; feed flow rate in ml h^{-1} was 30, 65, 110 or 157. Runs were performed in series at a constant temperature, two different glucose concentrations and four flow rates, obtaining the steady state concentration profiles inside the fermenter for each case. Six series were carried out, each one lasting around 15—20 days.

Analytical methods. Glucose and ethanol were determined by HPLC, using columns packed with Bio-Rad Aminex A-7 resin and water as eluent (pH = 7.0) at a flow rate of 1 ml min^{-1} and 80° C, in a HP-1090 Liquid Chromatograph with a refractive index detector. Lactose was used as internal standard.

Cell enumeration and viability. Cell concentration was estimated either by colony counts (spread plate technique in malt extract agar) or by direct microscopic enumeration in a Burker-type chamber. The latter method was also used to determine the cell viability by using the methylene blue staining method

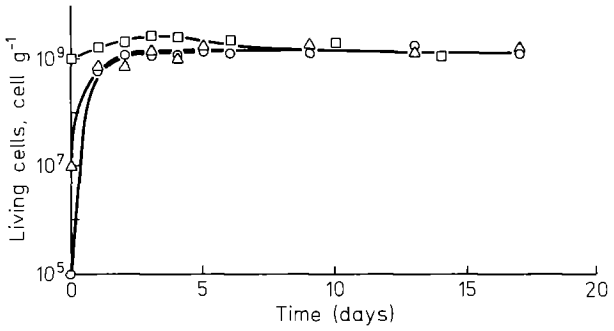

Fig. 1. Cell growth during fermentation inside the gel beads for various initial cell loadings (in number of living cells g^{-1} of gel): □, 10^9; △, 10^7; ○, 10^5

(Lee et al. 1981). When this determination had to be made in the gel beads, about 5 of them were soaked in 10 ml of 1% sodium citrate solution and shaken for 10 min at 30° C. In this way the beads were dissolved and cells could be counted by the previously explained methods.

Light microscopy of the gel beads. In order to determine the distribution of cells inside the gel beads, thin transverse sections were required for direct microscopic observation. This was achieved by the following method: the gel beds were fixed by immersion in a 2.5% gluteraldehyde solution in 0.1 M cacodylate buffer at pH = 7.4 with 2% $CaCl_2$ and 5% sucrose, for 4 h at 4° C, washed twice in cacodylate buffer with 2% $CaCl_2$ for 30 min and postfixed in 1% OsO_4 solution for 2 h at room temperature and in the dark. Following postfixation, beads were washed twice in cacodylate buffer with 2% $CaCl_2$ and dehydrated in a series of progressively more concentrated ethanol-water mixtures (30%, 50%, 70%, 90% and 100% ethanol) and then in propylene oxide, always at 4° C and for 30 min each. Finally, the dehydrated beads were enbedded in Durcapan epoxy resin (Fluka) for 12 h at 55° C. Once cold, the specimen blocks were cut in sections of 2 μm thickness by means of an Jeol Jum-7 ultramicrotome and examined by phase contrast microscopy.

Results and discussion

Bead properties

When beads were incubated in feed medium in a shaking-bath it could be observed that growth was very important in the first two days; thereafter the living cell concentration in the beads remained steady around 1.5 10^9 cells per gram of gel beads (Fig. 1), irrespective of the initial cell loading. In fact this was the maximum cell level observed in these beads, and after the second day it could be seen that the excess of cells grown in the beads was released from them. As an example of how cell distribution in the beads changed during incubation, two details of a bead slice just after immobilization (10^7 cell g^{-1} of gel initial cell loading) and after one day's incubation are shown in Figs. 2 and 3 respectively. The increase in cell density is remarkable, as well as the fact that it is more pronounced in the outer layer of the bead, as previously reported by other authors (Wada et al. 1980; Nagashima et al. 1984).

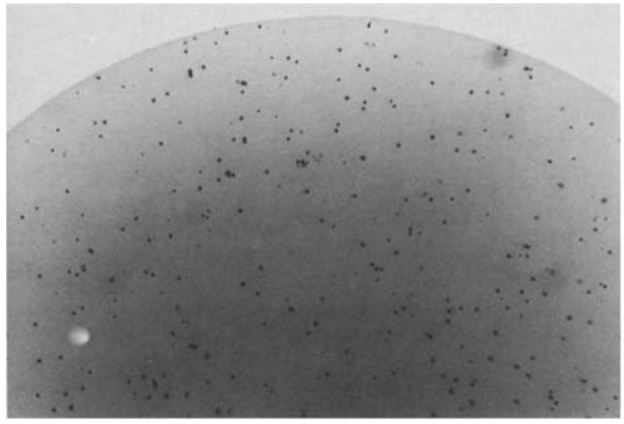

Fig. 2. Detail of transverse section of a gel bead just after immobilization (initial cell loading: 10^7 cells g^{-1} of gel)

Continuous fermentation

The performance of the continuous fermenter was determined by the measurement of steady-state concentration profiles along it for every set of conditions. In Fig. 4 typical global results are presented for one case and some examples of concentration profiles are given bellow. For each series of continuous fermentation runs in the packed-bed reactor, the cell concentration in the gel beads was determined before the experiment and at the end of it, measured at three different reactor heights: bottom, middle and top. As a

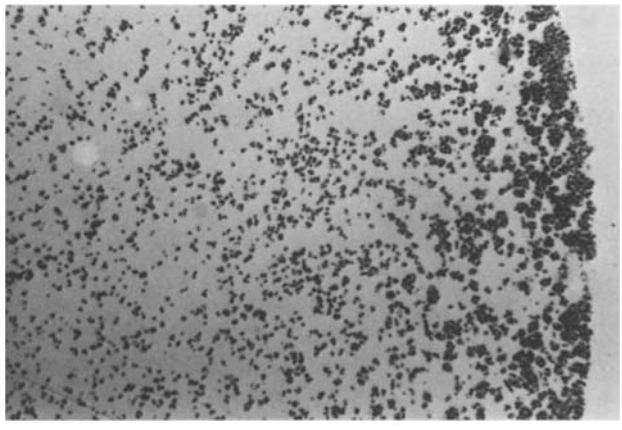

Fig. 3. Detail of a transversal section of a gel bead after one day fermentation (initial cell loading as in Fig. 2)

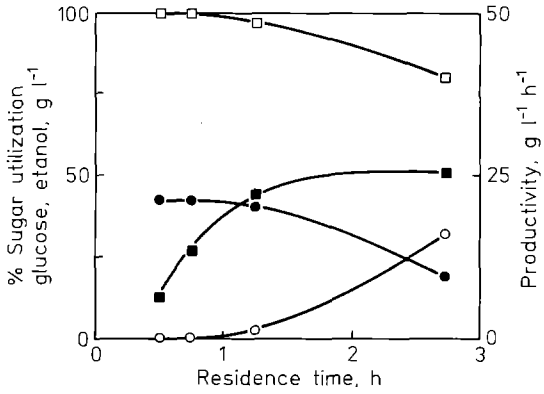

Fig. 4. Continuous fermentation results at various residence times for a 95 g l^{-1} glucose feed concentration and 30° C, ● ethanol concentration in the effluent, g l^{-1}; ○, glucose concentration in the effluent, g l^{-1}; □, percentage of sugar utilization; ■, ethanol productivity, g l^{-1} h^{-1}. Residence time is calculated on liquid volume basis and productivity on total volume fermenter basis

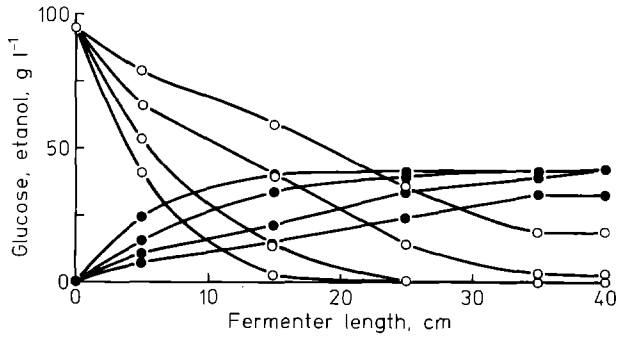

Fig. 5. Continuous fermentation profiles at 30° C. S_0 = 95 g l^{-1} and increasing flow rates (ml h^{-1}): (1) 30, (2), 65, (3) 110, (4) 157. ○, glucose concentration, g l^{-1}; ●, ethanol concentration, g l^{-1}

consequence of packed-bed characteristics no determination could be made during the experiments. The initial cell concentration was always around 1—2 10^9 cells g^{-1} of gel and its viability near 100%. The cell concentration and viability in the beads at the end of each fermentation series are shown in Table 1.

These results show that cell viability decreases with temperature and also from the bottom to the top of the fermenter. This second point agrees with the observed fermentation activity which, as can be seen from the concentration profiles in the fermenter, was always greater near the bottom, even when there were significant sugar concentrations in the rest of the fermenter (see Figs. 5 and 6). This is due to less favourable conditions for fermentation in the top section of the reactor (approximately from 20 cm of height to the end, 40 cm): a higher ethanol concentration, greater CO_2 accumulation (a major drawback of this type of fermenter) and O_2 exhaustion. This situation is inherent to the packed-bed fermenter and has also been pointed out by other authors who have studied internal concentration profiles (Chotani and Constantinides 1984; Gencer and Mutharasan 1981; Sitton and Gaddy 1980; Tyagi and Ghose 1982).

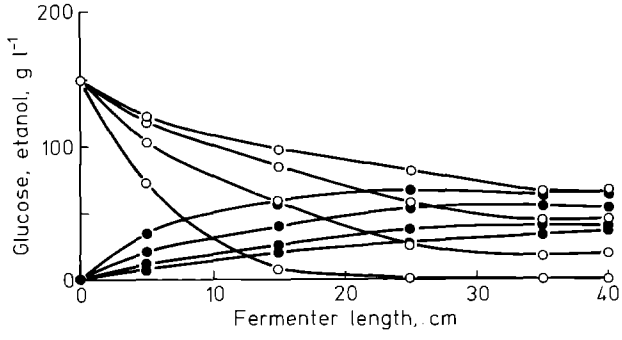

Fig. 6. Continuous fermentation profiles at 30° C. S_0 = 150 g l^{-1} and increasing flow rates (ml h^{-1}): (1) 30, (2) 65, (3) 110, (4) 157. ○, glucose concentration, g l^{-1}; ●, ethanol concentration, g l^{-1}

Table 1. Living cell concentration (LCC in cells g^{-1} of gel) and percentage viability (VB) in the tubular fermenter after continuous fermentation experiments

Experimental conditions			Living cells concentration (LCC) and viability (VB)					
T (°C)	S_0 (g/l)	Time (days)	Bottom		Middle		Top	
			LCC	VB	LCC	VB	LCC	VB
25	75/100	(25)	9.4 10^8	77.0	5.5 10^8	77.5	7.2 10^8	67.5
25	150/200	(23)	2.8 10^9	82.0	2.5 10^9	67.6	1.6 10^9	70.2
30	71/ 95	(18)	1.0 10^9	40.0	5.8 10^8	25.0	4.6 10^8	29.0
30	150/185	(25)	5.1 10^8	28.5	4.9 10^8	20.0	3.4 10^8	17.3
35	75/100	(18)	1.3 10^9	46.0	3.5 10^8	15.0	3.2 10^8	13.0
35	150	(13)	5.7 10^8	33.5	7.5 10^7	6.0	3.4 10^7	2.0

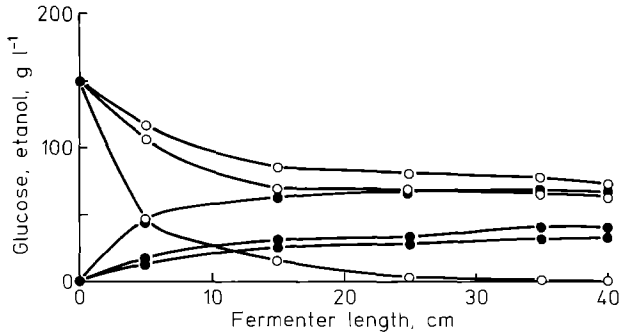

Fig. 7. Continuous fermentation profiles at 35° C. $S_0 = 150$ g l^{-1} and increasing flow rates (ml h^{-1}): (1) 30, (2) 65, (3) 110. ○, glucose concentration, g l^{-1}; ● ethanol concentration, g l^{-1}

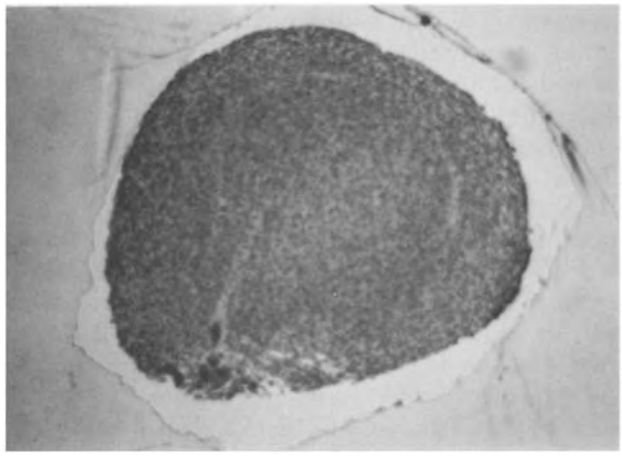

Fig. 9. Transverse section of a gel bead of the middle fraction of the fermenter after a continuous experiment

The viability decrease with temperature is dramatic at 35° C, especially when the glucose concentration is up to 150 g l^{-1}, and in fact the fermentation activity stopped at the second run (see Fig. 7). This indicates a limit of operation for the yeast strain used in the present study.

Regarding the final cell concentration at the bottom of the fermenter, it was about the same as that at the begining of the experiment in most cases, indicating that the loss in viability was compensated by cell proliferation in the beads. For the middle and the top fractions the living cell concentration usually decreased, but especially for those cases where the temperature effect was higher (30° C and 35° C).

When the transverse sections of the particles at different heights were observed microscopically (Figs. 8 to 10), clear differences were found. For the bottom section, cell density in the outer layer was much higher than in the rest of the particle (see Fig. 8), while this was not the case in the rest of the fermenter. Moreover, cell density in the top section seemed lower than in the rest of the column. Both observations are interesting and agree with the evolution in cell concentration and fermentative activity within the reactor: cell growth was greater in the more active section, where the diffusional limitations were clear and caused non-uniform cell distribution, while both factors were less important for the less active sections (middle, Fig. 9, and top, Fig. 10).

In conclusion, both microscopic observation of carrageenan gel beads and determination of cell concentration and viability are useful methods for characterizing the activity of immobilized cells in a packed-bed reactor and their results are

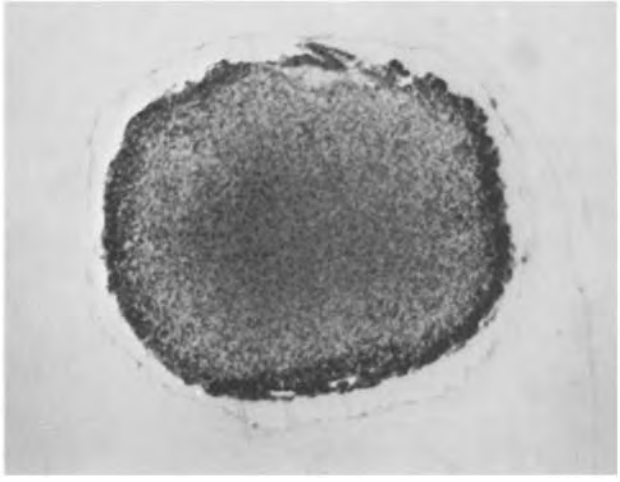

Fig. 8. Transversal section of a gel bead of the bottom fraction of the fermenter after a continuous experiment

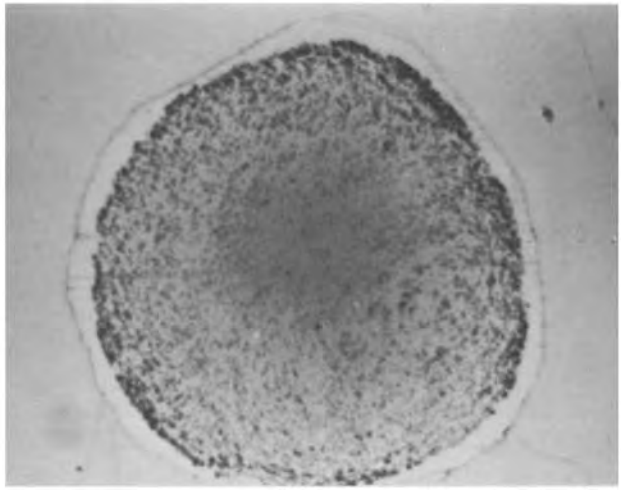

Fig. 10. Transverse section of a gel bead of the top fraction of the fermenter after a continuous experiment

in good agreement with the concentration profiles in the fermenter. A clear diffusion problem is observed for the bead size employed in this work, suggesting that smaller beads should have higher cell density throughout and therefore more efficiency. It seems evident that the packed-bed configuration, although easy to run, is not the best because only a limited fraction of the reactor works efficiently. Finally, the technique of bead-inclusion in Araldite for their microscopic observation seems particularly interesting as it allows thin slices to be obtained; in the near future these could be treated by an image processor to obtain quantitative data of cell distribution thereby improving our understanding of immobilized cells.

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