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Use of K_La as a Criterion for Scaling Up the Inulinase Fermentation Process

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ABSTRACT

The scale-up of inulinase production in aerated cultures of *Candida kefyr* DSM 70106 was studied, taking into account the criterion of maintaining K_La constant. The culture was carried out batchwise, in a 15-L fermentor, with K_La varying from 25 to 199 h^{-1}. The highest inulinase production was attained with an initial K_La value of 46 h^{-1}. A large scale fermentation (300-L fermentor) was performed using identical culture medium conditions. The responses obtained for the bench and scaled-up experiments showed similar behaviors, and the results were, respectively, 0.60 and 0.58 $U \cdot mL^{-1} \cdot h^{-1}$ for productivity and 43.0 and 41.5 $U \cdot mL^{-1}$ for activities.

Index Entries: Scale-up; inulinase; K_La; fermentation; *Candida kefyr*.

Nomenclature: $Y_{x/s}$, $\Delta X/\Delta S$; productivity (Pr), $\Delta P/\Delta t$; $\mu_{x(max)}$, maximum specific growth rate obtained from the slope of $\ln(x)$ as a function of time in the cell growth logarithmic phase; $U \cdot g_{cel}^{-1}{}_{(max)}$, specific enzymatic activity.

INTRODUCTION

Fructose has drawn much attention owing to its organoleptic properties and its sweetening power *(1)*. This sugar is generally obtained in the form of syrup, by isomerization of glucose coming from maize hydrolysis, which normally involves the activities of several enzymes *(2)*. An alternative to this process is the enzymatic hydrolysis of inulin, a polyfructosan composed of a linear chain of D-fructofuranosides.

Inulinase (2, 1 β-D-fructanfructanohydrolyse EC 3.2.1.7) is the enzyme that hydrolyzes inulin *(3)*. It is mainly produced by yeasts, such as *Candida kefyr*, *Kluyveromyces fragilis*, *Kluyveromyces marxianus*, *Debaryomyces cantarelli*, and *Pichia polymorpha*; filamentous fungi, such as *Aspergillus niger*, *Panaeolus papillonaceus*, and *Penicillium* spp. *(4–7)*; and plants like *Cichorium intybus (8)*. Manzoni and Cavazoni *(9)* studied inulinase production with four different yeasts and observered that *C.*

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kefyr had high potential for producing this enzyme, since it reached enzymatic activity levels comparable to those of the best strains of the other species tested. By using a 14-L capacity fermentor, the same authors obtained inulinase by a batch process under pH and temperature conditions similar to those of fermentations conducted in a 1-L fermentor, but with significant variations in agitation speed.

The industrial application of inulinase will only be viable if this enzyme is available in large quantities at competitive market prices *(10)*. The enzyme obtained on a large scale should present levels of activity, substrate conversion efficiency, and productivity equal to those obtained on a bench scale. Definitions of the most adequate microorganism and cultivation medium, as well as specification of the agitation and aeration conditions, are necessary for feasible scale-ups of fermentative processes *(11)*.

Extensive literature can be found on scale-up criteria of aerobic fermentative processes, such as power transmitted per unit of liquid volume, pumping capacity, or peripheral velocity of the turbines, the latter being closely related to the shear in the bioreactor. However, most of this is based on the same studies and correlations made and proposed between 1940 and 1950 *(12)*. When neither the microorganism nor the enzyme structure is damaged by shear and the dissolved oxygen can determine the cultivation yield, as it happens to inulinase from *C. kefyr* DSM 70106, the use of volumetric coefficient of oxygen transfer (K_La) as a criterion for scale-up of aerobic fermentative processes is quite appropriate. In this case, mass-transport processes are dependent on scale and account for scale-up problems *(13)*. K_La measures the performance of oxygen transfer from the gaseous to the liquid phase. Oxygen is very important for synthesizing products and biomass, since it is involved in the metabolism of the microorganism *(13–16)*. The aim of this work was to verify the criterion of maintaining a constant volumetric coefficient of oxygen transfer for scale-up during the cultivation of *C. kefyr* DSM 70106.

MATERIALS AND METHODS

Microorganism and Culture Media

In order to prepare the inoculum, *C. kefyr* DSM 70106 yeasts were transferred to three 1000-mL Erlenmeyer flasks containing 333 mL of activation medium previously sterilized at 121°C for 30 min. The flasks were incubated at 30°C for 24 h in a rotatory incubator (120 min^{-1}). After this, the contents of the three flasks were added to a 15-L fermentor containing 9 L of the following medium: $MgSO_4 \cdot 6H_2O$ (0.05 $g \cdot L^{-1}$), KH_2PO_4 (0.30 $g \cdot L^{-1}$), urea (2.25 $g \cdot L^{-1}$), $CaCO_3$ (0.01 $g \cdot L^{-1}$), peptone (6.5 $g \cdot L^{-1}$), yeast extract (2.8 $g \cdot L^{-1}$), and inulin (10.0 $g \cdot L^{-1}$). The cultivation conditions were: pH 5.0, 30°C. K_La of 43 h^{-1} (agitation speed of 120 min^{-1} and aeration at 1.0 vvm).

The cultivation for obtaining extracellular inulinase enzyme on a large scale was carried out in a 300-L fermentor containing 200 L of the same medium. The pH and temperature conditions for the cultivation were the same as those used for the 15-L fermentor. The K_La of 43 h^{-1} was obtained with 100 min^{-1} agitation and a 0.8 vvm aeration ratio. Samples were collected periodically to follow the variation of cell concentration (X), fructose content, total reducing sugars (TRS), and inulinase activity (P).

Enzymatic Activity Measurement

In order to determine the inulinase enzymatic activity, 0.20 mL of enzyme solution was mixed with 0.80 mL of 4.0% (w/v) buffered inulin solution (0.10*M* acetic acid–acetate buffer, pH 5.0) in a test tube and maintained at 50°C for 10 min. The reaction was stopped by immersing the test tube in boiling water. The amount of fructose formed was determined by Boeringer enzymatic assay. One inulinase unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol of fructose/min under test conditions.

Cell Mass Determination

The cell concentration values of the cultures were obtained by using a calibration curve to correlate optical density (OD) with dry weight ($g.L^{-1}$).

TRS Determination

The content of TRS was determined by a method described by Renscher *(17)*.

Kinetic Parameter Calculation

The specific cell growth rate (μ_x) was calculated as described by Le Duy and Zajic *(18)*. The substrate-to-cell conversion factor, defined as $Y_{x/s}$, was calculated as the ratio of cellular mass to substrate consumed ($\Delta X/\Delta S$). The productivity ($P = \Delta P/\Delta t$) was calculated as the ratio of the inulinase activity variation to the cultivation time (50 h).

Initial $K_L a$ Determination

The values of initial $K_L a$ (volumetric coefficient of oxygen transfer) were determined in the fermentation medium before the inoculum addition, as described by Wise *(19)*. This method estimates the $K_L a$ value, as a function of agitation and aeration conditions, based on the oxygen dissolution rate in culture medium. By combining different agitation and aeration values, the $K_L a$ value was regulated within the 25–199 h^{-1} range.

RESULTS AND DISCUSSION

Fermentative processes for inulinase production were carried out in two fermentors, with 15- and 300-L volumes. These fermentors had systems for automatic control of pH and monitoring of dissolved oxygen concentration. For the fermentation conditions on both scales to be as similar as possible, the $K_L a$ parameter was used as a criterion for process scale-up while maintaining all the other fermentative parameters equal and constant.

Figures 1 and 2 present the variations of the enzymatic activities and of cell and substrate concentrations in the 15- and 300-L fermentors, as a function of time. Figure 3 shows that the cell concentration vs time curves had similar behaviors for both fermentation scales. However, Fig. 3 also displays a dephasing in the behaviors of the specific rates of cell growth, which was more pronounced during the initial 6 h of the fermentative process.

The logarithmic growth phase was determined by the logarithmic curve of the cell growth as a function of time *(20)*. Several linear regressions of the experimental values were made, and the one that gave the best coefficient of linear corre-

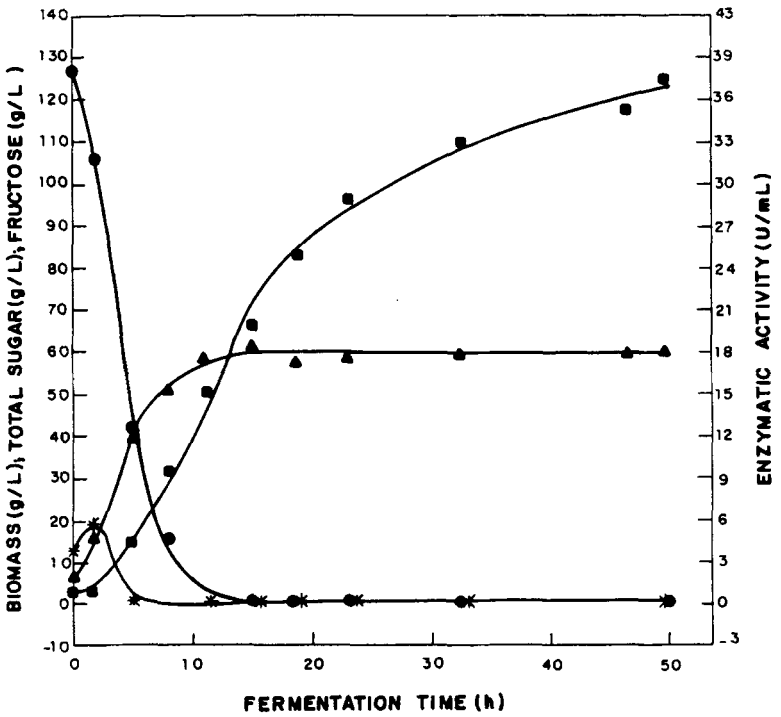

Fig. 1. Biomass (▲), total reducing sugar (●) and fructose (*) concentrations, and enzymatic activity (■) as a function of time during the cultivation of *C. kefyr* DSM 70106 in a 15-L fermentor.

lation (r) was used to define the exponential growth phase. From this point, it was noticed that the 15-L fermentation initiated its exponential growth 1 h after inoculation, whereas the 300-L fermentation started its exponential growth 2 h after the addition of the inoculum. Although this difference was small in relation to the total period of fermentation (50 h), it may have been responsible for the variation in the specific growth rate time curves.

According to Allais et al. *(21)*, the utilization of inulin as carbon and energy sources by a certain microorganism implies inulinase synthesis. Parekh and Margaritis *(10)* also demonstrated that the inulinase production by *K. marxianus* was induced by inulin. Looten et al. *(22)* studied the influence of several substrates on inulinase production and, in addition to confirming the catabolic repression by fructose, detected the production of this enzyme by *Clostridium acetobutylicum* when only inulin was used as a carbon source. The same characteristics of induction by inulin and repression by fructose were observed by Derycke and Vandamme *(23)* in *A. niger* cultivations.

As can be seen in Figs. 1, 2, and 4, for fermentations on bench and large scales, inulin (measured as TRS) was gradually hydrolyzed in the medium during cell growth, its total consumption occurring soon after the yeast had entered the cell growth stationary phase. The maximum specific rate of substrate consumption (Fig. 5) occurred during the cell growth exponential phase. This fact was also verified by Elyachiou et al. *(24)*. The shapes of the specific rate substrate consumption curves indicate that the 300-L fermentation tends to have smaller specific rates than does the 15-L fermentation.

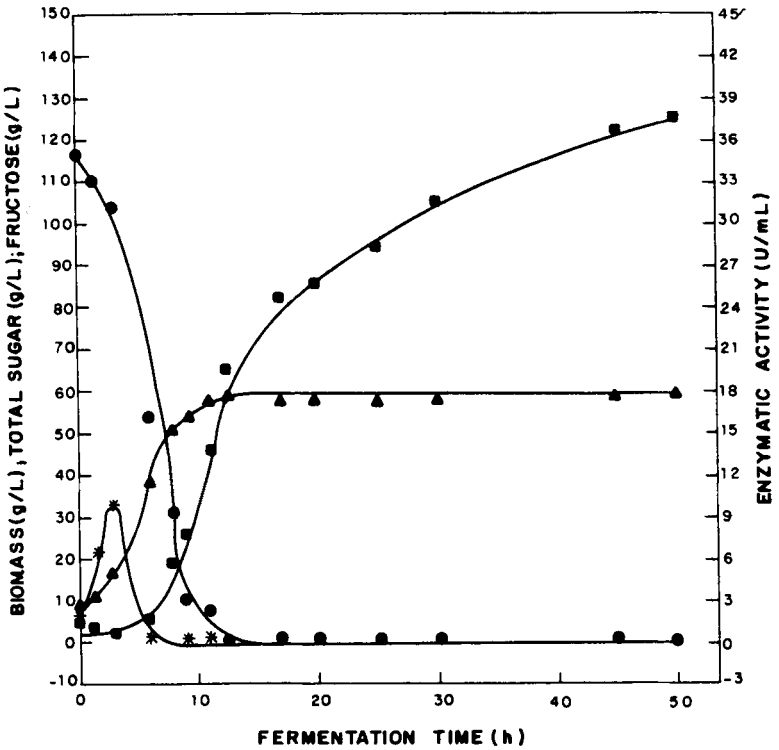

Fig. 2. Biomass (▲), total reducing sugar (●) and fructose (*) concentrations, and enzymatic activity (■) as a function of time during the cultivation of *C. kefyr* DSM 70106 in a 300-L fermentor.

Figure 4 also shows that fructose concentration remained low or zero during fermentation on both scales. During cultivation, Elyachiou et al. *(24)* also observed low accumulation of fructose in the medium, indicating that this monosaccharide was used by the cells immediately after its release by inulin hydrolysis.

When culturing *A. niger* in a medium containing inulin as a carbon source, Derycke and Vandamme *(23)* noticed that this polysaccharide caused inulinase to be produced mainly in extracellular form, with at most 15% of the total activity bound to the cell. Figure 6 depicts the increase in activity of inulinase obtained extracellularly as a function of time. In the initial cultivation phase, the enzyme synthesis suffered repression owing to fructose coming from the inulin hydrolysis during the sterilization stage. The same catabolic repression effect was found by Looten et al. *(22)* when cultivating *C. acetobutylicum* to obtain inulinase.

Figure 6 shows that, throughout the cultivation period, *C. kefyr* DSM 70106 produced extracellular inulinase. Considering the beginning of the stationary growth phase (t = 10 h approximately) and the end of the fermentation process (t = 50 h), the enzymatic activity increased from about 15 to 37 $U \cdot mL^{-1}$. This suggests that more than 50% of the inulinase produced was bound to the cell at the end of the cell growth exponential phase.

Inulinase production in both cultures paralleled cell growth. However, in the stationary phase, there was also an enhancement of enzymatic activity. This same behavior was reported by Manzoni and Cavazzoni *(25)*. This phenomenon can be analyzed better by comparing the microbial growth specific rate curves (Fig.

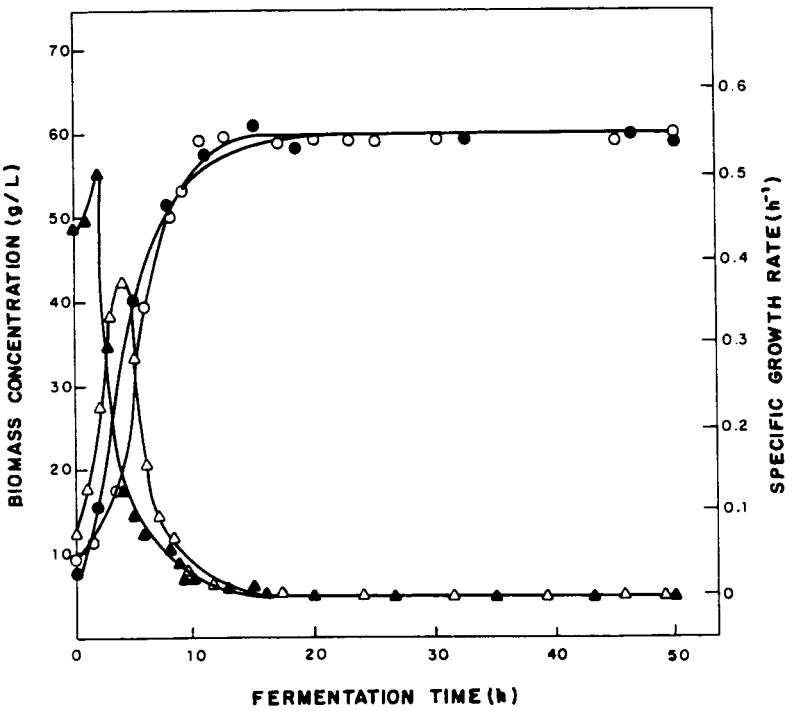

Fig. 3. Biomass (●,○) and specific rates of cell growth (▲,△) in 15- and 300-L fermentors as a function of time during the cultivation of *C. kefyr* DSM 70106.

3) with those of inulinase production (Fig. 6). Even in the stationary phase with microbial growth ($\mu_x = 0$), the inulinase production specific rate remains constant near 0.05 h^{-1}. Thus one can affirm that there is extracellular inulinase formation, even without microbial growth. According to Drent et al. *(26)*, this activity probably originated by cell breakage. In the present work, the cell breakage phenomenon was not clearly evident, leading to the assumption that enzyme was released by the cell by means of some other mechanism. Derycke and Vandamme *(23)* also observed this phenomenon while cultivating *A. niger* to obtain inulinase. These authors attributed this fact to the gradual release of enzyme still bound to the cell, the intracellular inulinase, to the culture medium. Their experiments to verify the intra- and extracellular enzyme properties showed that the optimum pH and temperature values of the two enzymes are identical, indicating similarity for these enzyme fractions. From a study of intra- and extracellular properties, Grootwassink and Fleming *(27)* verified that the inulinases are indistinguishable as far as their physicochemical properties and metabolic activities are concerned. They concluded that it is possible that the extracellular inulinase comes from intracellular inulinase released from its original position, possibly the periplasmatic space or the cellular wall.

Several studies have been conducted to correlate inulinase synthesis to microbial growth. When cultivating *Flavobacterium multivorum* LCB4 to obtain inulinase, Allais et al. *(21)* demonstrated that enzyme production was not associated with cell growth. Opposite results were found by Elyachiou et al. *(24)*, when cultivating *Anthrobacter* sp, and by Parekh and Margaritis *(10,28)* when culturing *K. marxianus*.

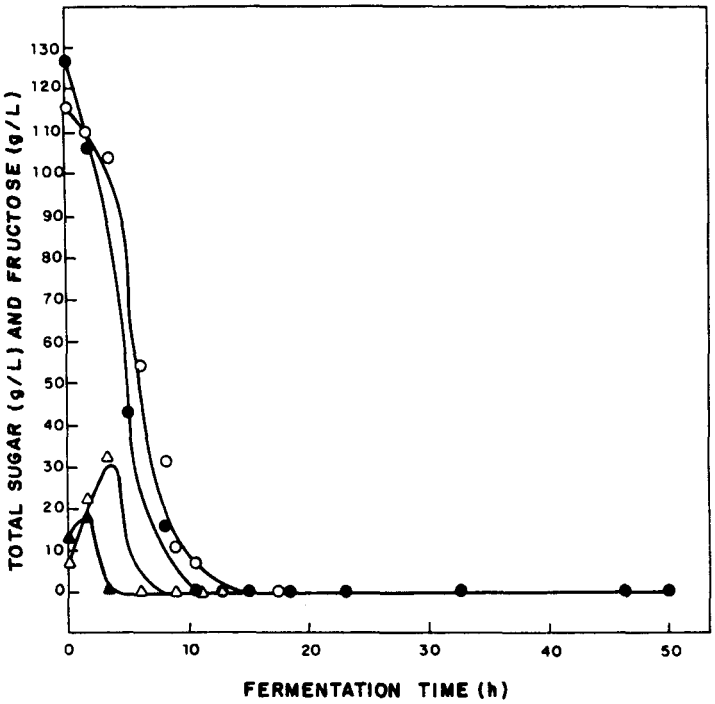

Fig. 4. Total sugar (○,●) and fructose (▲,△) concentrations in 15- and 300-L fermentors as a function of time during the cultivation of *C. kefyr* DSM 70106.

The dissolved oxygen as a function of fermentation time on bench and large scales was monitored, and its values are displayed in Fig. 7. During the exponential growth phase, the dissolved oxygen contents were reduced until reaching minimum values, indicating their immediate consumption by yeast right after transfer to the medium. Although the culture entered the cell growth stationary phase after 10 h of fermentation, the total consumption of the dissolved oxygen took approx 10 more hours. This was probably owing to the fact that microorganisms, which were still active, were utilizing oxygen for survival *(14)*.

With *K. fragilis*, Grootwassink and Fleming *(27)* noticed no change in the biomass and enzyme yields when the degree of saturation of dissolved oxygen varied from 2.5 to 40%. This indicates that the critical oxygen concentration value for forming the enzyme is lower than 2.5%. Since this level is so low, the authors suggest that the fermentation for producing inulinase could support cell densities much higher than those employed without affecting the yeast viability. In addition, the low critical dissolved oxygen requirement is beneficial for the process, since the driving force can be increased by operating at low dissolved oxygen.

Table 1 presents data obtained from bench- and large-scale fermentations for inulinase production. The parameter values—substrate-to-cell conversion factor ($Y_{x/s}$), productivity (Pr), cell concentration at the end of the fermentation ($X_{(max)}$), enzymatic activity ($U \cdot mL^{-1}$), and specific enzymatic activity ($U \cdot g_{cel}^{-1}$)—show that $K_L a$ usage as a scale-up criterion is quite appropriate, considering that the differences in the curve profiles throughout the cultivation are acceptable for fermentative processes. According to Oosterhuis and Kossen *(13)*, the similarity of these

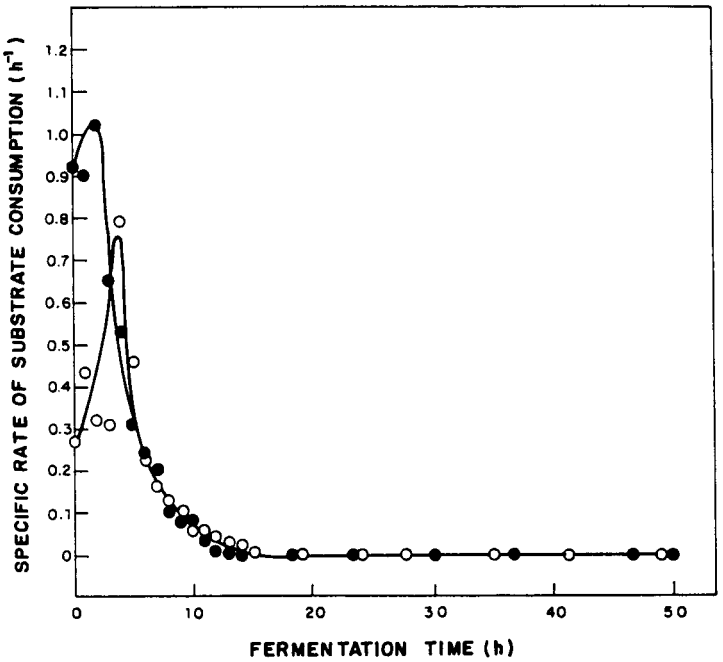

Fig. 5. Specific rate of substrate consumption as a function of time, from fermentations carried out in 15-L (●) and 300-L (○) fermentors.

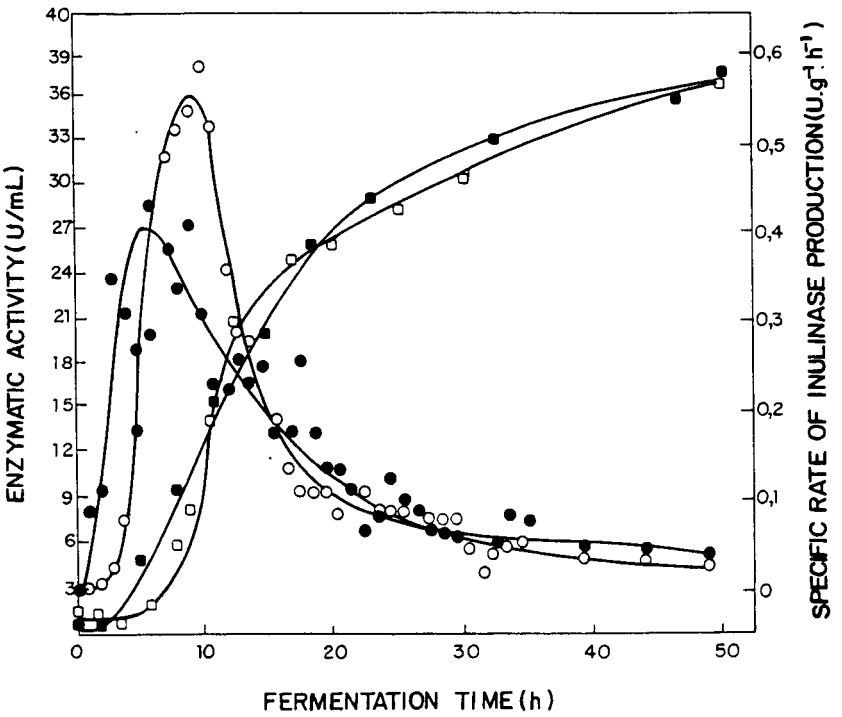

Fig. 6. Enzymatic activity (■,□) and specific rate of inulinase production (●,○) in 15- and 300-L fermentors as a function of time during the cultivation of *C. kefyr* DSM 70106.

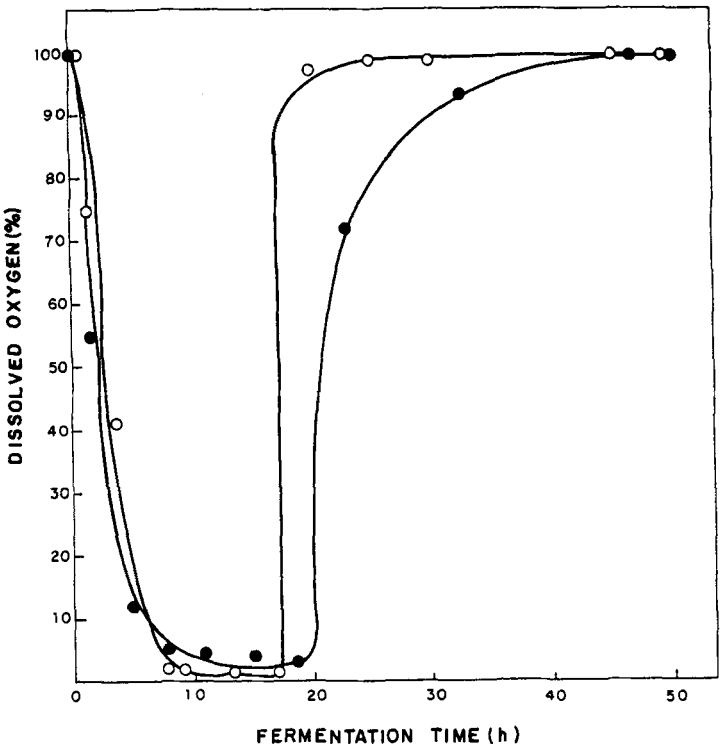

Fig. 7. Dissolved oxygen concentration as a function of time during the cultivation of *C. kefyr* DSM 70106 in 15-L (●) and 300-L (○) fermentors.

parameters on both cultivation scales is directly influenced by fluid dynamics. The fact that the medium viscosity was maintained constant during cultivation may have been the main cause of scale-up success.

Many studies on inulinase enzyme production have been published. In spite of this, detailed comparisons of their results are not feasible since the methods of enzyme activity determination are so variable. Considering only those studies containing small variations in these methods, one finds that the enzymatic activities are very low, varying from 0.2 to 7.2 $U \cdot mL^{-1}$ *(27,29)*. Higher enzymatic activity values were attained in experiments conducted by Manzoni and Cavazzoni *(9)*. Cultivating *C. kefyr* and *K. cicerosporus* in a 14-L fermentor containing 10 L of medium using pure inulin as a substrate, they obtained extracellular inulinase with 24.6 and 32.0 $U \cdot mL^{-1}$, respectively, after 6 d of fermentation. Later, these same researchers *(25)* cultivated *K. cicerosporus* having the *Helianthus tuberosus* extract as an inulin source. They found that this raw material led to an extracellular inulinase activity of around 96.9 $U \cdot mL^{-1}$, after 7 d of fermentation. Considering only 50 h of cultivation, they obtained an activity of about 38 U/mL^{-1}.

Taking into account that the analysis conditions (50°C/pH 4.7/5% inulin/15 min) used by Manzoni and Cavazzoni *(9)* furnish enzymatic activity results that are higher than the activities expected to be measured on an identical sample by the conditions used in our study (50°C/pH 5.0/4% inulin/10 min), a comparison of their results with ours was undertaken. In this way, it can be affirmed that the enzymatic activity of 37 $U \cdot mL^{-1}$ obtained after 50 h of cultivation with a K_La value of 43 h^{-1} can be considered equal or higher to the one obtained by the cited authors.

Table 1
Kinetic Parameters and Inulinase Production Data Obtained from Cultivations Carried Out in 15- and 300-L Fermentors

Parameters	Fermentor volume, L							
	15							300
K_La, h^{-1}	25.0	35.0	39.0	43.0	70.0	164.4	199.0	43.0
Aeration ratio, vvm	1.0	1.0	0.5	1.0	1.0	1.0	1.0	0.8
Agitation, min^{-1}	180	100	120	120	150	300	500	100
$Y_{x/s}$, $g_{cel} \cdot g^{-1}_{sub}$	0.47	0.48	0.47	0.47	0.48	0.48	0.49	0.49
Pr, $U \cdot mL^{-1} \cdot h^{-1}$	0.51	0.60	0.68	0.73	0.65	0	0	0.71
$X_{(max)}$, $g \cdot L^{-1}$	5.8	5.7	5.8	5.9	5.8	5.8	5.9	5.9
$U \cdot mL^{-1}_{(max)}$	25.5	30.2	34.0	37.5	32.6	0	0	36.7
$U \cdot g^{-1}_{cel\ (max)}$	4396.6	5298.2	5862.1	6356.0	5260.7	0	0	6220.3
$\mu_{x,(max)}$, h^{-1}	—	—	—	0.23	—	—	—	0.24

Manzoni and Cavazzoni *(9)* considered their enzymatic activity highly promising and without equal in the literature, justifying in this way the continued study of extracellular inulinase production.

CONCLUSIONS

The production of inulinase by *C. kefyr* DSM 70106 has been shown to be dependent on the coefficient of transferred oxygen. At values of $K_L a$ above 70 h^{-1}, inhibition of enzyme production by dissolved oxygen is found. A reduction of enzymatic activity indicating a limitation on the production of enzyme by oxygen was observed for $K_L a$ values below 43 h^{-1}. The adoption of $K_L a$ as a criterion for scale-up of the fermentation process for enzyme production was shown to be adequate, since it resulted in similar values for productivity and the substrate/cell conversion factor.

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