

# Osmotic Inhibition of Free and Immobilized *K. marxianus* Anaerobic Growth and Ethanol Productivity in Whey Permeate Concentrate

M. C. Dale, A. Eagger & M. R. Okos

Biochemical & Food Process Engineering, Department of Agricultural Engineering, Purdue University, West Lafayette, IN 47907, USA

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*The effects of high concentrations of whey permeate on a lactose fermenting yeast, K. marxianus, were studied using both free and immobilized cell reactors.*

*Examination of substrate and product inhibition data from the literature suggests that a simple linear osmolality model may account for both the substrate and product inhibition of anaerobic yeast growth and ethanol productivity for this yeast and others. The osmolality of sweet whey permeate concentrate (SWPC) and acid whey permeate concentrate (AWPC) solutions were determined and fitted to a simple empirical model. The batch free cell growth rates and immobilized cell growth and productivity in these solutions was then determined. Batch cell growth was found to be 85% inhibited at osmolalities of 2.2 os/kg in both AWPC and SWPC, although the maximal specific growth rate for SWPC was 2.6 times higher than for AWPC. Immobilized cell growth inhibition patterns were different between AWPC and SWPC. An 80% inhibition in effluent cell density was noted at an osmolality of 1.3 os/kg with AWPC, while with SWPC, this level of inhibition was not reached until 1.9 os/kg. The cell growth of the immobilized cells was more strongly inhibited than the free cells at higher solution osmolalities.*

*The effect of osmolality on immobilized cell productivity as measured by gas evolution rates was also examined. A 66-85% inhibition of productivity was noted at 2.4 os/kg (31% solids) with AWPC, while SWPC was only 30-60% inhibited at 2.2 os/kg (40% solids). The immobilized cells were more tolerant to high osmolalities if the osmolality was increased slowly over time, indicating adaptation by the immobilized cells. The productivity of immobilized cells was less inhibited by high osmolality than free cell productivity as reported by other researchers.*

## NOTATION

$K$  Numerical constants  
 $K_{ev}$  Linear constants for osmotic inhibition of productivity (osmol/kg)

$K_{\mu}$  Linear constant for osmotic inhibition of cell growth (osmol/kg)  
 $P$  Product concentration (ethanol) (g/litre)  
 $P_{max}$  Product concentration (ethanol) where growth or productivity is totally inhibited (g/litre)  
 $S$  Substrate concentration (lactose, sucrose) (g/litre)

Corresponding author: M. C. Dale. Tel: 317 494 1195; Fax: 317 496 1115.

|              |                                                                                 |
|--------------|---------------------------------------------------------------------------------|
| $S_0$        | Threshold sugar concentration where osmotic inhibition is first noted (g/litre) |
| $x_s$        | Percentage solids (w/v) in solution                                             |
| $\epsilon_0$ | Initial osmolality at which inhibition is first noted (osmol/kg)                |
| $\mu$        | Specific growth rate (g cell/g cell h)                                          |
| $\mu_{\max}$ | Maximum (uninhibited) specific growth rate (g cell/g cell h)                    |
| $\nu$        | Specific productivity (g eth/g cell h)                                          |
| $\nu_{\max}$ | Maximum (uninhibited) specific productivity (g eth/g cell h)                    |

## INTRODUCTION

Each organism has a maximal, optimal, and minimal water activity for growth and productivity. In general, microbes will tolerate a lower minimal water activity if all other environmental parameters (temperature, pH, nutrient mix) are near optimal for that organism. Most bacteria and yeast have a growth limiting minimal  $a_w$  of around 0.91 (5.2 os/kg). But osmophilic yeasts can grow slowly in media with  $a_w$ s as low as 0.62–0.65 (23.9–26.5 os/kg).<sup>1,2</sup> If the osmotic environment of a solution is higher than the natural internal osmotic pressure within the cells, there will be a net flux of water from the cell. The osmolality of a solution is an additive property of the osmolality of the various solutes (assuming that solute interaction effects are small), which makes osmolality a useful function to work with in fermentation systems. The osmolality of a glucose solution being fermented to ethanol increases as one mole of sugar is converted to two moles of ethanol. Jones and Greenfield<sup>1</sup> note that in batch yeast fermentations exponential growth cycle cessation was often noted at a point in the fermentation where solution osmolality began to increase.

Glycerol is used by *Saccharomyces cerevisiae* yeast as an osmoregulatory internal product. The osmolality inside the cell is regulated to be slightly higher than the external environmental osmolality. Under conditions of minimal  $a_w$  environments (maximal solution osmolality) glycerol production in the cell can not keep up with the leakage rate through the cell membrane, plasmolysis results and there is not cell growth.<sup>3</sup> Kenyon *et al.*<sup>4</sup> noted a fourfold increase in glycerol levels when a CSTR was operated at an osmolality calculated as 1.6 os/kg versus a 0.33 so/kg solution. Osmotolerant yeasts have a higher natural internal osmotic

potential (3.5 os/kg for *Saccharomyces rouxii*) allowing higher osmotic environments to be tolerated before osmotic inhibition starts. Osmotolerant yeast also seems to be less permeable to polyols and thus better able to retain these internal osmoregulatory products, with the major polyol produced by *S. rouxii* being arabitol rather than glycerol.<sup>5</sup>

The onset of osmotic inhibition of ethanol productivity for yeast seems to begin at osmolalities above the natural internal osmolality of the cell. In a study with two strains of *S. cerevisiae*, osmotic inhibition of growth began at an osmolality of 1.0 for both strains tested, falling linearly to zero at an osmolality of 3.0 for the more osmotolerant strain while the less tolerant strain showed plasmolysis at slightly under 2.0 os/kg.<sup>6</sup> With *Saccharomyces uvarum* yeast, a 400 g/litre sucrose solution (2.4 os/kg initial osmolality) was noted to inhibit maximum batch productivity by 62% as compared to a 250 g/litre sucrose solution (initial osmolality of 1.07 os/kg). Altering the osmotic environment of a 10% sucrose solution in a non-growth medium with increasing levels of sorbitol gave maximum ethanol productivity in a fermentation broth with an initial osmolality of 0.63 and final osmolality of 1.37. The total ethanol productivity was reduced by 46% and the final cell viability reduced by 80% in a fermentation with an initial osmolality of 2.9 and final osmolality of 3.3 os/kg.<sup>7</sup> It was also noted that cells retained ethanol internally for longer periods of time during batch fermentations and showed a higher final inter-cellular ethanol concentration as the osmolality of the environment increased. Another investigation of osmotic influences on yeast using an 18% glycerol solution to give an initial broth osmolality of 2.84 showed a 54% decrease in productivity at 6 h as compared to a solution with an initial osmolality of 0.78.<sup>8</sup>

In a recent study on the effects of osmolality (using glycerol to modify the osmotic potential) on free and immobilized *Zymomonas mobilis* at a high operational temperature,<sup>9</sup> it was determined that immobilized cell growth was more strongly inhibited than that of free cells by increasing osmolality, but that the same inhibition pattern was noted. It was postulated that the immobilized cells, due to their high concentrated density on the column packing, might be experiencing an osmolality of 1.5 os/kg higher than the bulk solution. The productivity of the immobilized cells was slightly more inhibited than the free cells at osmo-

lalties of under 5.0 os/kg, but the immobilized cells showed higher productivity than free cells at osmolalities over 6 os/kg. Ethanol inhibition of cell growth and productivity was much stronger than inhibition due to only solution osmolality, suggesting specific ethanol inhibition at 40°C for this organism.

If new 'plug flow' type immobilized cell reactor-separators implementing simultaneous production separation are used, ethanol product inhibition limits are lifted allowing more concentrated feeds to be fed to the reactor.<sup>10-12</sup> The new limiting factor on maximum feed concentration to the bioreactor becomes the ability for the fermenting organism to withstand the high osmotic potential of the feed. If the fermentation of high concentrations of substrate is desired, it will be critical to know the effects of high solution osmolality on cell growth and productivities. If growth rates are more inhibited than productivities by high osmolalities, feed concentration to a bioreactor might be increased to limit cell growth and the concomitant reactor clogging in immobilized cell type reactors. Reactor clogging by cell overgrowth can be a problem in trickle flow type immobilized reactors.<sup>11,12</sup> Osmotic inhibition may also be rate limiting for stirred tank type reactors incorporating simultaneous ethanol removal. Feeds such as molasses and whey have substantial mineral components, and these build up in the reactor. Cysewski and Wilke,<sup>13</sup> show that a certain rate of bleed is required to keep the non-fermentables in the 'vacu-ferm' reactor contents from building up to the point where osmotic inhibition reduced reactor performance. In the similar concept 'Biostil' reactor<sup>14</sup> (where a side stream from the reactor is stripped of ethanol and returned) osmotolerance of the yeast, rather than ethanol tolerance is most critical to reactor performance. The objective of this investigation was to determine the effect of high concentrations of whey permeate, both sweet and acid types, on cell growth and ethanol productivities of a lactose fermenting yeast using batch and immobilized cell reactors.

Certain strains of lactose utilizing yeasts have been screened and determined to be more tolerant to high levels of feed lactose. Moulin *et al.*<sup>15</sup> selected two strains as being able to ferment 30% permeate with 50 and 55% of the lactose being converted to ethanol. Vienne and Stockar<sup>16</sup> showed that strain *Kluyveromyces marxianus* 665 was able to ferment an 18% whey permeate solu-

tion faster and with a slightly higher efficiency than other strains, including the strains used by Moulin *et al.*<sup>15</sup> Strain *K.m.* 665 was selected for this research.

## MATERIALS AND METHODS

### Strains and permeate

*K. marxianus* NRRL 665 was obtained from the Northern Regional Research Laboratory in Peoria, IL. Dried sweet whey permeate was purchased from Wisconsin Dairies Coop, concentrated sweet whey permeate was provided by Swiss Valley Farms of Maquoketa, IA, and concentrated acid whey was provided by Dairy Farms Co. of Orville, OH.

### Determination of osmolality

A vapour pressure osmometer, Westcor model 5100CXR, was used for the determinations of osmolalities of molasses solutions. This osmometer is able to determine osmolalities of solutions up to a maximal determination of about 3.5 os/kg. Whey permeate solutions were tested, and lactose solutions were also prepared to check the reliability of the readings as well as to obtain osmolalities beyond those given in reference texts.<sup>17</sup>

### Batch studies

Flasks or tubes of various concentrations of reconstituted dried whey permeate, concentrated sweet whey permeate, and concentrated acid whey permeate were prepared. An inoculum of 1% of aerobically grown cell suspension was added to each batch reactor. These were grown in still culture with the cell density determined using the optical density at 660 nm as a function of time.

### Immobilized cell reactor (ICR) studies

A short 'differential' type columnar reactor was constructed of a 2 in ID glass tube packed with a piece of terry cloth and spiral wound with a nylon spacer (Fig. 1). The reactor was run in a trickle bed, gas continuous mode. It had an internal packed volume of 115 ml, and a liquid hold-up of 30 ml. The feed rate to the reactor was held constant at 60 ml/h for a liquid residence time of 30 min. *K. marxianus* 665 was grown in a flask of sterile nutrient medium (10 g/litre lactose, 3 g/litre yeast extract, and 3 g/litre malt extract). After 24 h growth in this medium, the yeast broth

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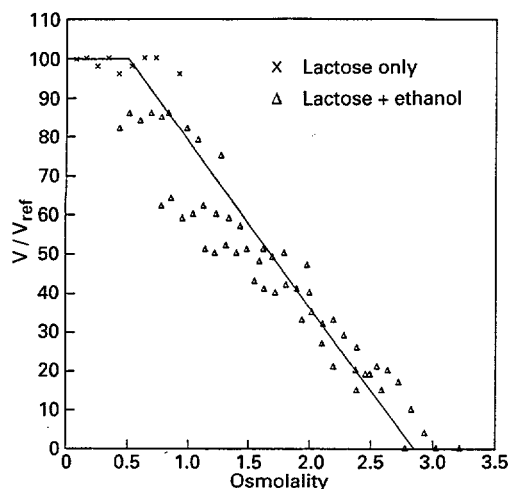


Fig. 2. Relative productivity of *C. pseudotropicalis* as a function of osmolality on lactose and ethanol (data from Moulin *et al.*<sup>15</sup>).

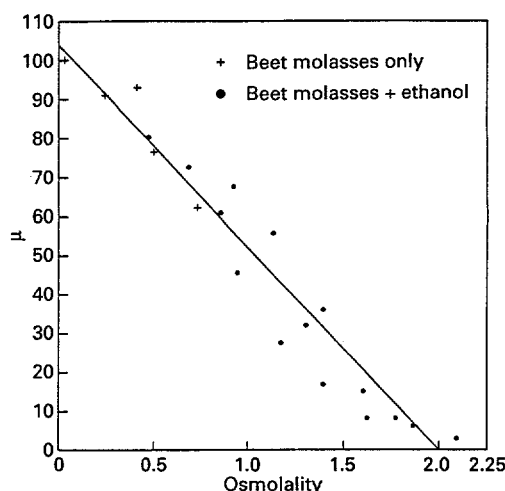


Fig. 4. Relative growth rates of *S. cerevisiae* as a function of osmolality (data from Letourneau and Villa<sup>18</sup>).

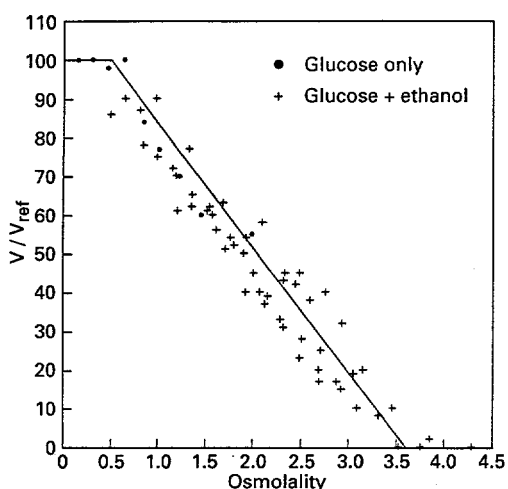


Fig. 3. Relative productivity of *C. pseudotropicalis* as a function of osmolality on glucose and ethanol (data from Moulin *et al.*<sup>15</sup>).

similarly transformed as shown in Fig. 4 with 0 os/kg for  $\epsilon_0$  and 2.0 os/kg for  $K_{\epsilon\mu}$ . Thus, both substrate and ethanol inhibition of growth and productivity are largely the osmotic phenomena for these yeast strains. Table 1 shows the correlation coefficients ( $r^2$ ) between the linear osmolality model and the more complex (up to five coefficients) models proposed by Moulin *et al.*<sup>15</sup> and Letourneau and Villa.<sup>18</sup> The fit by the simple linear osmolality model is nearly as good in all cases.

The results shown here suggest that both ethanol (product) and sugar (substrate) inhibition of anaerobic yeast cell growth and productivity are

largely osmotic phenomena. This observation should be helpful in the design and operation of ethanol reactors. The inhibition effects of inerts (salts, glycerol, lactic acid, and other non-fermentables) can be substantial with complex substrates like molasses, whey (both sweet and acid types), and in reactors in which broth is evaporated or recycled.<sup>13,14</sup> Osmolality in such a reactor can be estimated if the composition of sugars, ethanol and inerts is known (allowing the osmolality to be determined), or the osmolality of the reactor may be monitored as a controlled variable even if the composition of inerts is unknown or complex. Measuring or estimating the solution osmolality will give a good prediction of the expected cell growth and productivity inhibition due to inerts, substrate and product.

There may, of course, be further toxic or inhibitory compounds in the broth with specific effects beyond osmotic. Ethanol has further toxic effects on yeast besides its osmotic inhibiting effects. The effects of ethanol on various yeast species has been examined by a number of researchers, and many models for ethanol inhibition of yeast suggested. The fact that (1) ethanol inhibition of cell growth and productivity increases with increasing temperatures, and (2) ethanol increases the cell death rate, are evidence that ethanol has toxic effects on the yeasts beyond osmotic inhibition. The toxic effects of ethanol (as distinguished from the osmotic inhibition effects) are particularly important for immobilized cell reactors, as Dale *et al.*<sup>19,20</sup> show that the live cell fraction of a dynamic immobilized cell population can be expected to

Table 1. Osmolality model for substrate and product inhibition of yeast

|                                    | Model                                                                                                                                                                                                                             | Solution                                | r <sup>2</sup> |
|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|----------------|
| Proposed                           | $v/v_{\max} = \left[ 1 - \frac{\varepsilon - \varepsilon_0}{K_{\varepsilon v}} \right]$ $K_{\varepsilon v} = 2.3$ $\varepsilon_0 = 0.5$                                                                                           | Lactose and ethanol <sup>15</sup>       | 0.929          |
| Moulin <i>et al.</i> <sup>15</sup> | $v/v_{\max} = \exp(-K_{L1}P)$ $-K_{L1} = 0.0157$                                                                                                                                                                                  | Lactose and ethanol <sup>15</sup>       | 0.924          |
| Proposed                           | $v/v_{\max} = \left[ 1 - \frac{\varepsilon - \varepsilon_0}{K_{\varepsilon v}} \right]$ $K_{\varepsilon v} = 3.1$ $\varepsilon_0 = 0.5$                                                                                           | Glucose and ethanol <sup>15</sup>       | 0.927          |
| Moulin <i>et al.</i> <sup>15</sup> | $v/v_{\max} = \exp(-K_{G1}P) \exp(-K_{G2}(S - S_0))$ $\exp[-K_{G3}(S - S_0)P]$ $K_{G1} = 9.4 \times 10^{-3}$ $K_{G2} = 3.5 \times 10^{-3}$ $K_{G3} = 1.9 \times 10^{-4}$ $S_0 = 100 \text{ g/litre}$                              | Glucose and ethanol <sup>15</sup>       | 0.969          |
| Proposed                           | $\mu/\mu_{\max} = \left[ 1 - \frac{\varepsilon}{K_{\varepsilon \mu}} \right]$ $K_{\varepsilon \mu} = 2.0$                                                                                                                         | Beet molasses and ethanol <sup>18</sup> | 0.950          |
| Letourneau and Villa <sup>18</sup> | $\mu/\mu_{\max} = \exp(-K_{S1}(S - S_0)) \left[ 1 - \frac{P}{P_m} \right]^{(K_{S2} + K_{S3}S)}$ $K_{S1} = 5.5 \times 10^{-3}$ $K_{S2} = 0.4$ $K_{S3} = 7.4 \times 10^{-3}$ $S_0 = 117 \text{ g/litre}$ $P_m = 70 \text{ g/litre}$ | Beet molasses and ethanol <sup>18</sup> | 0.959          |

decrease over time if growth rates are not higher than cell death rates.

#### Whey permeate osmolality

The osmotic potentials of various whey solutions were determined as shown in Fig. 5. Whey permeate has a higher osmolality than lactose, due to the salt fraction, and AWPC has a higher osmolality than SWPC. Acid whey with a pH of 4.5 is a by-product of cottage cheese production, and has more lactic acid than sweet type whey which has a pH of 5.3–5.5. Reconstituted dried SWPC was found to demonstrate a higher osmolality than

fresh SWPC at the same solids concentration. The reason for this difference in osmolality may be due to precipitation of some proteins and/or higher molecular weight salts in the drying process. Lactose osmolality to 24% solids (the solubility limit at room temperature) was determined. Our determinations at lower concentrations were close to literature values<sup>17</sup> as shown in Fig. 5.

The data on solution osmolality as a function of solids were fitted empirically to a simple two parameter model based on percentage solids ( $x_s$ ):

$$\varepsilon_s = [k_1 x_s]^n \quad (4)$$

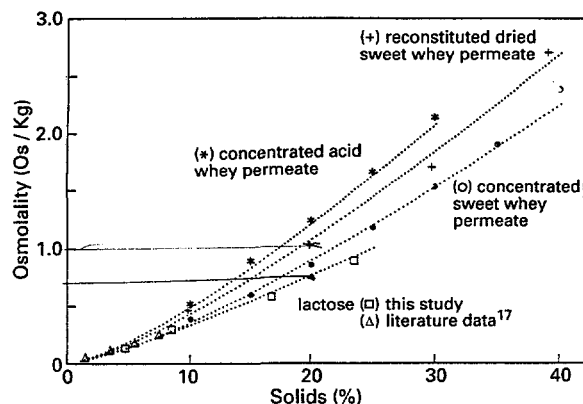


Fig. 5. Osmolality of various whey permeate solutions as a function of percentage solids. (Dotted lines show the fit of the data to eqn (8) using the constants from Table 2.)

Parameters  $k_1$  and  $n$  were determined as shown in Table 2 for the various solutions and the resulting fits are shown in Fig. 5 as the dotted lines.

#### Batch growth studies

Six replications of batch growth curves were performed for acid whey permeate at concentrations of 15, 20, 25 and 30% solids, and sweet whey permeate at 15, 20, 25, 30, 35 and 40% solids. Sample growth curves are shown in Figs 6 and 7, with the averaged maximal exponential specific growth rates plotted against osmolality in Figs 8 and 9. It can be seen that the growth rates are very inhibited above an osmolality of 2.0 with both sweet and acid whey showing a total inhibition of growth at about 2.4 os/kg if the inhibition curve is extrapolated to zero growth rate. The maximal specific growth rate with sweet whey (at 15% solids) was 2.6 times higher than AWPC ( $\mu$  of  $0.396 \text{ h}^{-1}$  for SWPC versus  $0.152$  for AWPC).

#### ICR cell growth and productivity

The productivity of a short differential immobilized cell reactor as a function of different feed concentrations was next determined. Cell densities in the reactor effluent were also monitored as an indicator of immobilized cell growth. If a steady state cell density in the reactor is assumed, then the effluent cell density can be used to estimate the number of cells grown.<sup>20</sup> The effects of AWPC concentration and osmolality on effluent cell density and relative productivity are shown in Figs 10 and 11. Cell growth of the immobilized cells was inhibited at 20% solids (1.34 os/kg), and there was no major difference in growth inhibition between when osmolalities were increased or

Table 2. Osmolality constants for various solutions (eqn (4))

|                                                       | $k_1$ | $n$  | $r^2$ |
|-------------------------------------------------------|-------|------|-------|
| Lactose                                               | 0.040 | 1.20 | 0.985 |
| Sweet whey permeate concentrate (SWPC)                | 0.046 | 1.33 | 0.993 |
| Reconstituted sweet whey permeate concentrate (RSWPC) | 0.053 | 1.32 | 0.990 |
| Acid whey permeate concentrate (AWPC)                 | 0.058 | 1.32 | 0.999 |
| Glycerol                                              | 0.121 | 1.23 | 0.998 |

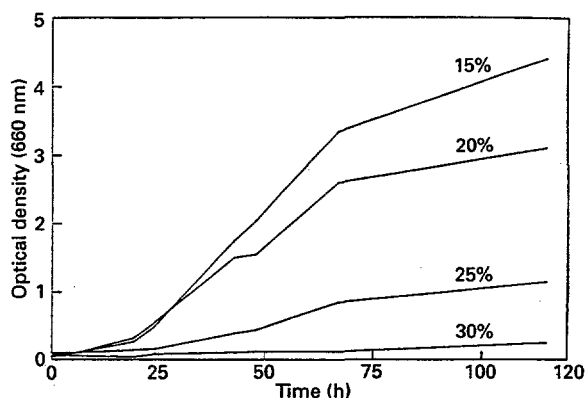


Fig. 6. Batch growth of *K. marxianus* in concentrated acid whey permeate at various concentrations.

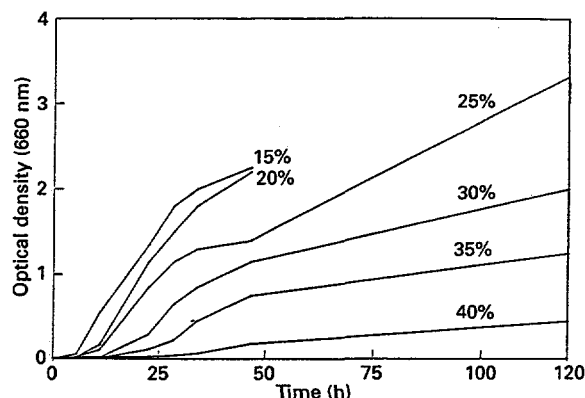


Fig. 7. Batch growth of *K. marxianus* in concentrated sweet whey permeate at various concentrations.

decreased with time. An 85% drop in effluent cell densities was noted as the feed concentration increased from 15 to 20% (0.92–1.34 os/kg) with little further inhibition as the osmolality was increased. Productivity was less inhibited than growth at higher osmolalities. There was a major difference in the degree of productivity inhibition when osmolality was increased with time rather than decreased. When the osmolality was

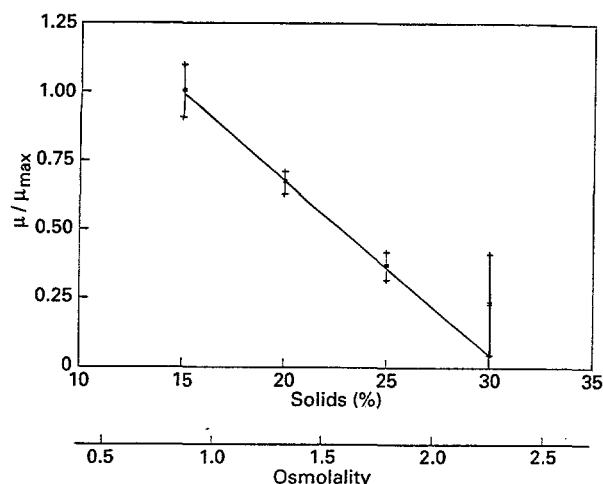


Fig. 8. Maximal batch growth of *K. marxianus* as a function of acid whey permeate concentration ( $\mu_{\max} = 0.152$ ).

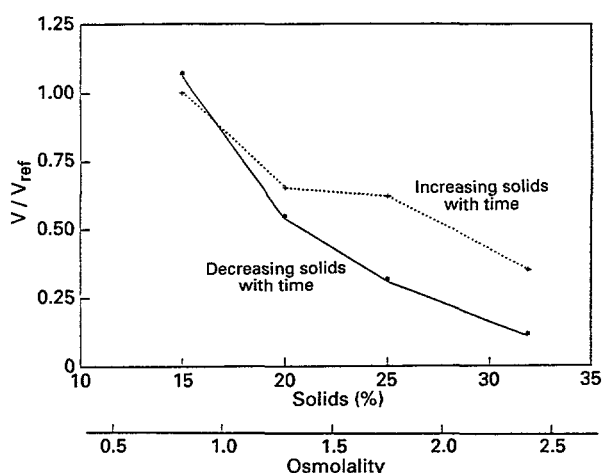


Fig. 11. Relative productivity of ICR as a function of acid whey permeate concentration.

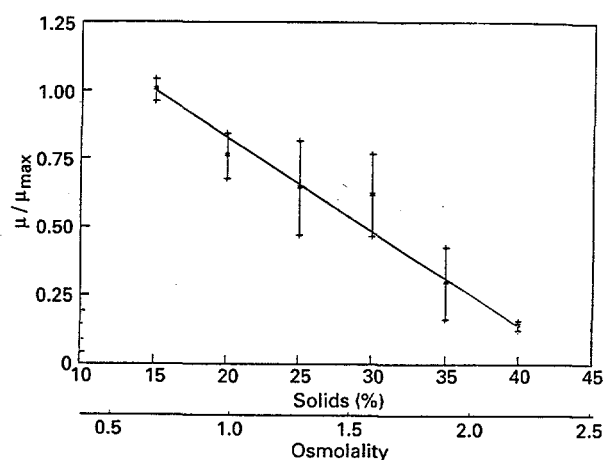


Fig. 9. Maximal batch growth rates of *K. marxianus* as a function of sweet whey permeate concentration ( $\mu_{\max} = 0.396$ ).

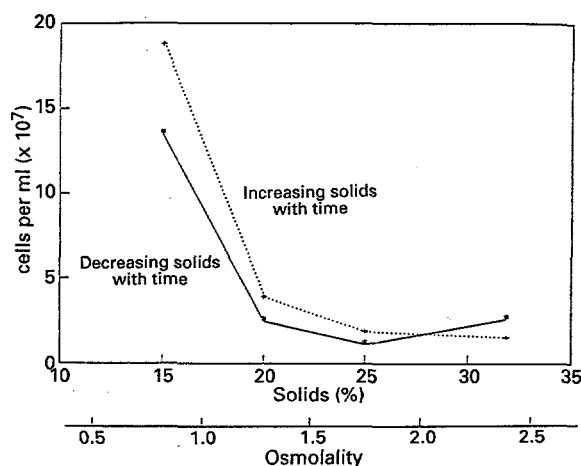


Fig. 10. Effluent cell density from the ICR as a function of acid whey permeate concentration.

increased from 0.92 to 2.4 slowly over the period of a week, the productivity dropped gradually to 35% of the original rates. But, when the osmolality was changed abruptly from basal 0.92 os/kg to 2.4 os/kg, productivity at 2.4 os/kg was only 13% of the basal rate.

With SWPC, effluent cell densities showed a difference in inhibition patterns between increasing and decreasing osmolality (Figs 12 and 13). The effluent cell density decreased fairly linearly between 1.3 and 1.8 os/kg when osmolality was increased with time. When osmolality decreased with time, a steep decline in effluent cell density was noted between 1.1 and 1.3 os/kg. Productivity patterns for SWPC were fairly similar when concentration increased or decreased with time, with much less inhibition noted at higher osmolalities than with AWPC. Productivity showed only a 15% decrease at 1.9 os/kg when concentration increased or decreased with time, inhibition was noted at osmolalities under 2.0 os/kg.

The effects of osmolality on the productivity of immobilized cells in AWPC and SWPC are compared to the effects noted by Moulin *et al.*<sup>15</sup> with free cells in a lactose-ethanol medium in Fig. 14. We see that the immobilized cells in SWPC seem to be less inhibited than the free cells, probably due to adaptation of the immobilized cells. The cells in the acid whey are more strongly inhibited by increasing osmolality than with the SWPC, indicating that there is some constituent in the AWPC which is inhibitory to the yeasts. The decreased productivity rates seen in concentrated AWPC are not due solely to osmotic effects. As acid whey is produced by the lactic acid ferment-

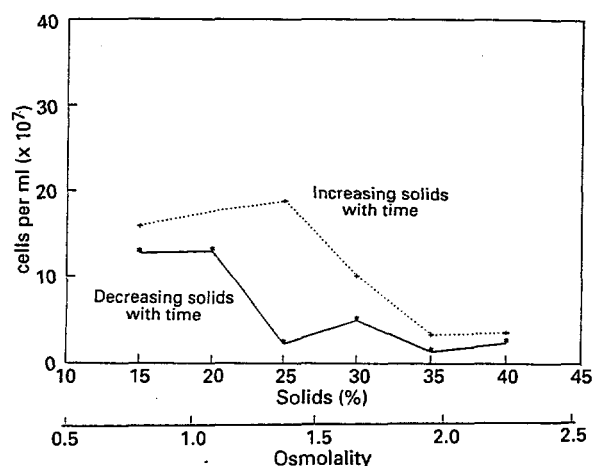


Fig. 12. Effluent cell density from the ICR as a function of sweet whey permeate concentration.

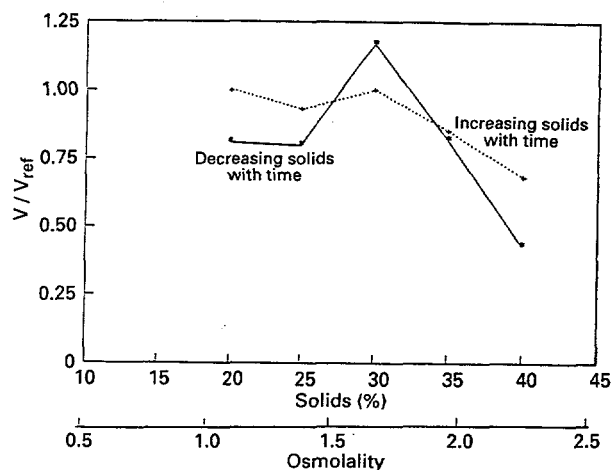


Fig. 13. Relative productivity of ICR as a function of sweet whey permeate concentration.

tation of milk during the cheese making process, it seems likely that there is some low level by-product(s) of the lactic acid fermentation that are inhibitory to yeast, such as acetic or propionic acids, and flavour compounds (diacetyl, acetaldehyde and acetoin). Volatile acids (acetic, propionic) generally account for about 2–3% of the total acid produced.<sup>21</sup> There is no doubt that the lactic acid fermentation of milk to cottage cheese or yogurt greatly improves the 'keeping' qualities of the fermented product, indicating the formation of inhibitory compounds. Lactic acid itself is not particularly inhibitory at the levels found in AWPC (5–20 g/litre) based on preliminary experiments performed in our laboratory (unpublished data).

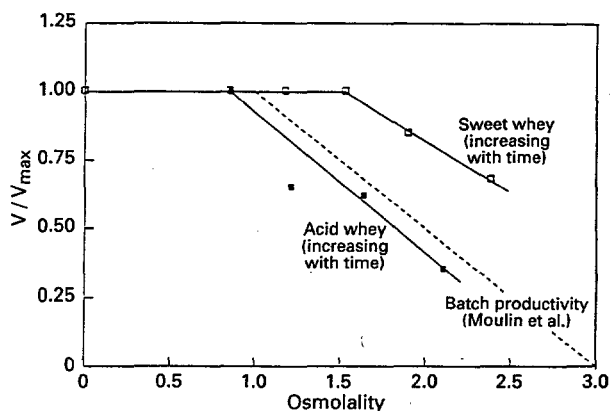


Fig. 14. Relative productivity of immobilized cells in AWPC and SWPC as a function of osmolality.

## CONCLUSIONS

The use of reactors incorporating product separation or the development of ethanol tolerant yeast strains will allow highly concentrated feeds to be fed to a bioreactor. In determining the effects of concentration or feed osmolality on yeast performance, it was determined that both substrate and product inhibition can largely be attributed to osmotic effects, with a linear decrease in productivity and growth noted as solution osmolality increases. Immobilized cells show less inhibition of productivity but higher inhibition of growth with increasing osmolality as compared to free cells. The growth of both free and immobilized cells is much more strongly inhibited than productivity by osmolality. Strong (80%) inhibition of free cell growth was noted at about 2.2 os/kg in both types of permeate, while strong inhibition of immobilized cell growth rates was noted at 1.3 os/kg with AWPC, and in SWPC at 1.8 os/kg. Free and immobilized cell growth and productivity were all significantly lower in acid type whey permeate concentrate as compared to sweet type whey permeate concentrate at the same solution osmolality.

In an immobilized cell reactor, excess cell growth can cause clogging, but lack of any cell growth will lead to reactor performance deterioration as cells die and are not replaced.<sup>20</sup> If a maximal concentration of feed is defined as that concentration which gives not more than 80% inhibition of cell growth and not more than 50% inhibition of productivity, a maximal feed concentration of sweet whey permeate concentrate of 35% can be determined, while for acid type WPC

a maximal feed concentration of 20% can be determined. The limits of feed concentrations for both feeds are due to cell growth rather than productivity limitations.

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**Appendix . B.**

*High Density Ferm. w/ Flocc. Yeast*  
Dale & Zhao (research rpt)