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The Promotion of Molasse Alcoholic Fermentation Using *Saccharomyces cerevisiae* in the Presence of γ-Alumina

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ABSTRACT

Using γ-alumina pellets, more than threefold increase of the ethanol productivity in the fermentation molasse has been obtained in the present work. Also, molasse fermentation in the presence of γ-alumina gave 78.4 g/L ethanol, ethanol yield factor 0.44 g/g, and conversion 89.4% at initial sugar concentration (ISC) 179.5 g/L, compared to 53.9 g/L, 0.30 g/g and 62.7% in its absence, respectively. Furthermore, it was found that γ-alumina reduces the activation energy Ea of fermentation. This inorganic material does not promote the fermentation of raisin extract.

Index Entries: γ-Alumina; alcoholic fermentation; molasse; promotion; raisin extract; *Saccharomyces cerevisiae.*

INTRODUCTION

Recently, considerable attention is given in Greece to the use of the inorganic support γ-alumina in ethanol fermentation, and a recent study specifically examined the immobilization of *Zymomonas mobilis* cells on the porous γ-alumina pellets (*1*). Additionally, in the frame of this work

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using free cells of *Z. mobilis* a promotion of the alcoholic fermentation carried out in synthetic media containing glucose in the presence of γ-alumina was observed. The same phenomenon in the ethanol fermentation of glucose using the traditional microorganism *Saccharomyces cerevisiae* (*2*) was also noticed. Furthermore, the continuous potable alcohol production by immobilized *Z. mobilis* on γ-alumina (*3*) using raisin extract, as well as the repeated batch fermentations using immobilized *S. cerevisiae* on γ-alumina pellets (*4*), have been reported. Likewise, it was found that kissiris, another porous mineral material, significantly increases the ethanol production rate of molasse fermenation employing *S. cerevisiae* (*5*). The improvement of the ethanol production rate and the reduction in the energy demand in the industrial alcoholic fermentation processes were the main aspects of the research made the last period, and especially after the oil crisis of 1973. For the increase in ethanol productivity, the use of the bacteria *Z. mobilis* (*6*), which ferments faster than yeast, as well as cell immobilization (*7–10*) were proposed. *Z. mobilis* (*11–12*) was also proposed for the energy demand reduction, because of its ability to grow in higher ethanol concentrations than the traditional *S. cerevisiae*. To the best of our knowledge, no material has been proposed for attainment of higher ethanol concentrations and the promotion of molasse fermentation employing *S. cerevisiae* cells, with the exception of the mineral kissiris (*5*). After the above presentation of the research made for the promotion of the alcoholic fermentation, a detailed study concerning the effect of γ-alumina pellets on the alcohol production rates, obtained in the fermentation of raw materials, is also necessary. The subjects of the present work were: (i) To study the influence of γ-alumina on the ethanol productivity achieved in molasse and raisin extract; and (ii) to examine the possibility of attaining higher ethanol concentrations.

METHODS

Materials

The γ-alumina Houndry Ho415 cylindrical pellets (with ca.5mm length, 2.5 mm diameter, 0.45 cm^3/g pore vol, and 1.40 m^2/g surface area) was used. The Greek sugar beet molasse was the raw material employed in this investigation without pretreatment. The pH was adjusted to 4.7 with sulfuric acid after dilution of molasse with tap water at the convenient °Be density (containing sacchrose 102 g/L, 147.1 g/l, 165.3 g/l, 179.5 g/l). KH_2PO_4 (0.5 g/L) was added and the whole was sterilized in an autoclave at 130°C for 15 min. Greek raisin, variety Trechumena, was employed for the preparation of raisin extract. Tap water and raisin at proportion obtaining raisin extract having a desired sugar concentration, were placed in a conical flask and heated for 4 h in water bath (72°C). The raisin extract was diluted with tap water at the appropriate °Be density (10, 14, and 18 °Be). Fructose and sucrose were food-grade as well as glucose suitable for microorganisms cultures. Invert-sugar was a mixture prepared from glucose and fructose in a proportion of 1:1 w/w. In the fermentation procedure, baker's yeast, *S. cerevisiae*, was supplied in cakes from bakeries.

Fermentations

In a 1-L beaker were placed 100 g γ-alumina pellets, 500 mL diluted and sterilized molasse, or 500 mL raisin extract or 500 mL synthetic media (containing 215 g/L of each of sugars glucose, fructose, invert-sugar, sucrose separately, 1 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$, 5 g/L $MgSO_4$, 2 g/L yeast extract), and 10 g wet wt *S. cerevisiae* cells. In order to compare the ethanol productivity obtained in the presence of γ-alumina with that in its absence, similar runs were carried out simultaneously. These experiments were made at 30°C, without stirring. The kinetics of fermentation were followed by measuring the °Be density at various time intervals. The pH of molasse and sucrose solutions during fermentation were adjusted at 4.7 value with sulfuric acid addition. Glucose fermentations in the presence and in the absence of γ-alumina were also carried out in 20°C parallels with those of 30°C. Likewise, glucose fermentations at 20°C were obtained in the presence of (a) γ-alumina pellets, (b) its powder obtained by a screen of 850 μm, and (c) powder achieved by a screen of 212 μm. In the case of the use of powder, fermentations were carried out after agitation by a magnetic stirrer.

Repeated Batch Fermentations

Before the fermentation end of the batch performed by the aforementioned method in the presence of γ-alumina, the liquid was decanted and the γ-alumina was transferred to another beaker containing 500 mL sterilized molasse (16.5 °Be density) and 10 g *S. cerevisiae* cells. Thus, a new fermentation was started and it was repeated seven times. The pH during the fermentation was adjusted as described above.

Determinations

Ethanol was determined using a gas chromatograph (Varian model 1400) equipped with an FI detector and a column (2 m length and 1/8 in id) filled with Porapac S. Nitrogen, with 99.99% purity, was used as the carrier gas. Its flow rate was 40 mL/min. The determination of ethanol made possible the calculation of the ethanol productivity, defined as the g of ethanol produced/L in 24 h, as well as the ethanol yield factor, defined as the ratio of g of ethanol produced/g of sugar used. Sugar determinations were performed using the anthrone test (*13*). Biomass was determined employing the optical density procedure at 700 nm. For pH measurements, a CFG D-6900 pH-meter was used.

RESULTS

Effect of γ-Alumina on Molasse Fermentation

S. cerevisiae cells and diluted molasse were mixed once with γ-alumina pellets as well as with the absence of it simultaneously. In the frame of this, batch fermentations were carried out to examine the promotional effect caused by the inorganic material on molasse fermentation. The experiments were performed at various relative high initial °Be densities, to study the promotional effect at °Be densities preferred in industry because of their influence on the reduction of the energy demand and cost. All experiments were performed at the same pH, γ-alumina content (1 g/5 mL), and the initial cell concentration (ICC). The kinetics of fermentations, made in the presence and in the absence of γ-alumina, were also studied in all experiments. The results are presented in Fig. 1. After the end of the fermentations, samples were analyzed for ethanol and sugar determination for the calculation of ethanol productivity, yield, and conversion. Related results are presented in Table 1. This table shows that γ-alumina pellets have a positive effect on the fermentation efficiency of *S. cerevisiae* in the case of molasse. All results show that this material causes an important increase in ethanol productivity. Specifically, the increase of the initial sugar concentration (ISC) augmented the differences in the fermentation time and ethanol productivity between the presence of γ-alumina and the absence of it. The 50% increase in productivity achieved at ISC 102 g/L is raised to 267% in the case of 179 g/L ISC. Using γ-alumina pellets in all ISC studied, conversion and yield are in an acceptable level. These parameters obtained with the absence of γ-alumina are in an unpractical level, except that achieved at low ISC. Moreover, the aforementioned inorganic material decreases the concentration of residual sugar. The later and the final ethanol concentrations achieved indicate that in the presence of this material, the fermentation of molasse takes place even at relatively higher ethanol levels. An inspection of Fig. 1 shows that in all cases studied, the presence of this material renders the kinetics of molasse fermentation faster. Likewise, yeast ferments efficiently at higher sugar concentration in the presence of γ-alumina in comparison with the absence of it, where the fermentation stopped at relatively high °Be level. Fermentations carried out by the γ-alumina pellets gave an increase of pH in molasse's fermentation broth. As a result of the great effect of pH on invertasse activity, it was adjusted to 4.7 during fermentation by the addition of sulfuric acid. Thus, the pH adjustment

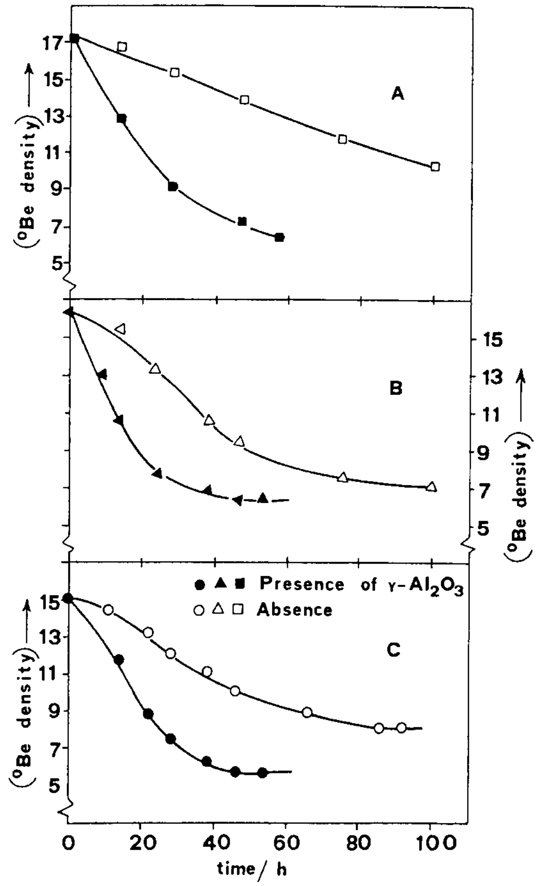

Fig. 1. Fermentation kinetics observed for molasse both in the presence and the absence of γ-Alumina at different initial sugar concentration (ISC). (■), (□) 179.5 g/L (A); (▲), (△) 165.3 g/L (B); (●) (○) 147,1 (C).

was made so that it does not pass the 4.9 pH value. The increase of pH observed in the fermentation of molasse by the presence of γ-alumina pellets are not concordant with the results obtained in glucose (2) fermentations in the presence of it. In this case, the pH remained relatively constant. Measurements of biomass during the fermentation showed that γ-alumina does not significantly increase the free cell concentration.

Repeated Batch Fermentations

All batches were carried out using the γ-alumina pellets employed in the first batch, the same initial °Be density of molasse, and similar ICC. The repeated batch fermentations were performed to examine the promotional effect of γ-alumina on the ethanol fermentation of molasse from batch to batch. The results of the first seven repeated batch fermentations are illustrated in Table 2 and Fig. 2. Table 2 indicates that the fermentation time, ethanol productivity, residual sugar, conversion, and yield to be relatively

Table 1
Effect of γ-Al_2O_3 on the Most Important Alcoholic Fermentation Parameters Obtained by the Use of Molasse

Initial sugar concentration, g/L	Fermentation time, h	Final ethanol concentration, g/L	Ethanol productivity, g/L/24 h	Residual sugar, g/L	Conversion, %	Ethanol yield factor, g/g
179.5	144 (57)[a]	53.9 (78.4)	9.0 (33.0)	66.9 (19.0)	62.7 (89.4)	0.30 (0.44)
165.3	101 (47)	59.2 (70.4)	14.1 (35.9)	22.8 (5.5)	86.2 (96.7)	0.36 (0.43)
147.1	86 (45)	50.7 (60.8)	14.1 (32.4)	33.2 (5.8)	77.4 (96.1)	0.35 (0.41)
102.0	15 (10)	43.6 (43.3)	69.8 (104)	5.5 (5.3)	94.6 (94.8)	0.43 (0.43)

[a]The numbers in parenthesis refer to fermentations carried out by the presence of γ-Al_2O_3.

Table 2
Effect of γ-Alumina on Ethanol Fermentation[a]

Repeated batch fermentation	Initial sugar concentration, g/L	Fermentation time, h	Final ethanol concentration, g/L	Ethanol productivity, g/L/24 h	Residual sugar, g/L	Conversion, %	Ethanol yield factor, g/g
1	165.3	70 (29)	56 (70.4)[b]	19.2 (58.3)	— (11.0)	— (93.3)	0.34 (0.43)
2	165.3	142 (40)	56 (70.4)	9.5 (42.2)	33.8 (13.8)	79.6 (91.7)	0.34 (0.43)
6	165.3	142 (38)	56 (71.2)	9.5 (45.0)	33.8 (13.8)	79.6 (92.1)	0.34 (0.43)
7	165.3	80 (39)	66.4 (75.2)	19.9 (46.3)	31.5 (11.7)	80.9 (92.9)	0.40 (0.46)

[a]Indicates the most important alcoholic fermentation parameters obtained in the repeated batch fermentations of molasse in the presence of the same amount of γ-Al_2O_3 pellets as compared with the absence of it.
[b]The numbers in parenthesis refer to fermentations carried out by the presence of γ-Al_2O_3.

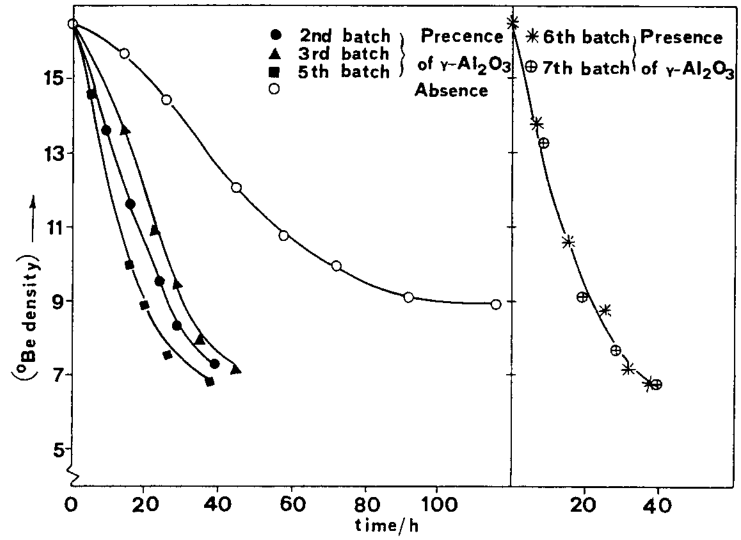

Fig. 2. Fermentation kinetics observed for molasse in repeated batch fermentations in the presence of γ-Alumina, as compared to the absence of it.

constant from batch to batch. Figure 2 shows the kinetics observed at the repeated batch fermentations using γ-alumina pellets, as compared with its absence. In all repeated batch fermentations, the same γ-alumina pellets renders the fermentation faster.

Effect of γ-Alumina on Raisin Extract Fermentation

Raisin is widely used in Greece as raw material for ethanol production employing fermentation by the traditional baker's yeast *S. cerevisiae*. The abundance in Greece of this raw material that is rich in invert-sugar is in contrast to molasse containing saccharose. Also, a positive effect on its ethanol production rate is of great economic importance for the countries employing raisin as raw material in the production of alcohol. Batch fermentations were performed in the presence of γ-alumina and in the absence of it at various °Be densities of raisin extracts. All fermentations were carried out at the same ICC, γ-alumina content, and initial pH. The initial pH used was the pH of raisin extracts, wich is 3.7. The kinetics of fermentations are illustrated in Fig. 3. The figure shows that γ-alumina pellets used in raisin extract has no significant effect on the ethanol fermentation rate in all initial °Be densities studied.

Effect of γ-Alumina on Fructose, Invert-Sugar, and Sucrose Fermentations

Fermentations of synthetic media containing fructose, invert sugar, and sucrose to examine their relationship with the effect of γ-alumina on

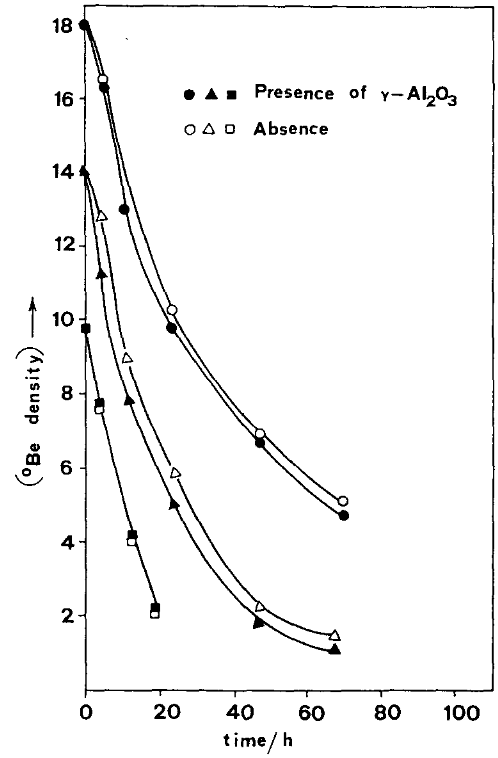

Fig. 3. Fermentations kinetics observed for raisin extract in the presence and the absence of γ-alumina at different initial °Be densities.

the fermentation of raw materials containing these sugars (molasse and raisin extracts), were also performed. Figures 4, 5, and 6 clearly show that γ-alumina renders faster the fermentation of fructose, invert sugar, and sucrose. In all cases, γ-alumina causes a decrease of final °Be density. This presumably indicates that in the presence of γ-alumina, fermentation takes place even at relatively high ethanol levels.

Effect of Temperature on Ethanol Fermentation Rate in the Presence of γ-Alumina

In order to investigate the effect of γ-alumina on activating energy in the case of alcoholic fermentation, kinetics at 20 and 30 °C in the presence and in the absence of γ-alumina were performed. Figure 7 illustrates that the presence of γ-alumina pellets causes an increase in the fermentation time 65%, and the absence 133% as the temperature of the fermentation drops from 30 top 20 °C. This means that the proportion of reaction speed constants at 30 and 20 °C K_{30}/K_{20} becomes lower in the case of the presence of γ-alumina pellets as compared with those of its absence. Taking into account the Arrhenhius equation, $\log(K_2/K_1) = E_a/2.303\,R\,\{(T_2-T_1)/T_2 \cdot T_1\}$,

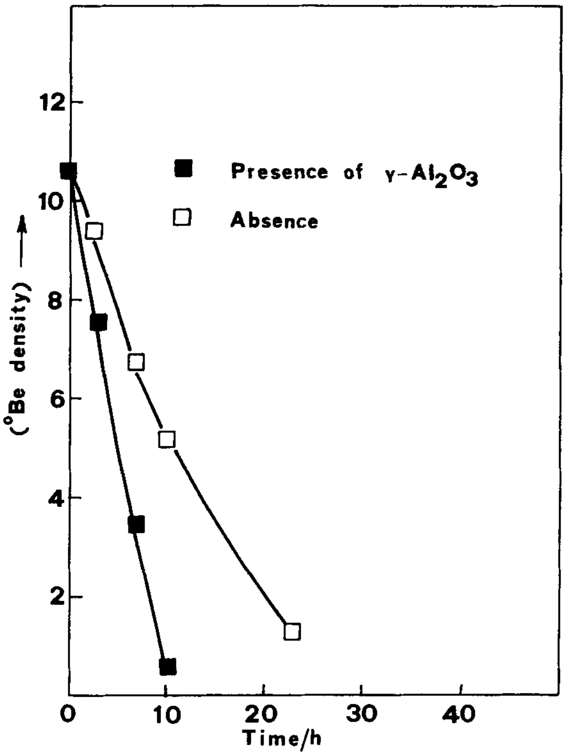

Fig. 4. Fermentation kinetics of fructose observed in the presence of γ-alumina as compared to the absence of it.

the activation energy E_a is reduced in the presence of γ-alumina pellets. The latter contributes to the idea that γ-alumina behaves as a catalyst or as a promoter of the catalytic activity of the enzymes involved in the process (*14*).

Effect of γ-Alumina's Particle Size on Ethanol Fermentation Rate

To examine the effect of particle size of γ-alumina, fermentations of synthetic media containing glucose, in its absence and presence in the form of different particle size were performed. Figure 8 shows that γ-alumina increases fermentation rate in all particle size used. Particle size of 850 μm was more effective.

DISCUSSION

The promotion of the synthetic media containing glucose fermentation by the γ-alumina pellets using *Z. mobilis* (*1*) and *S. cerevisiae* (*2*) was

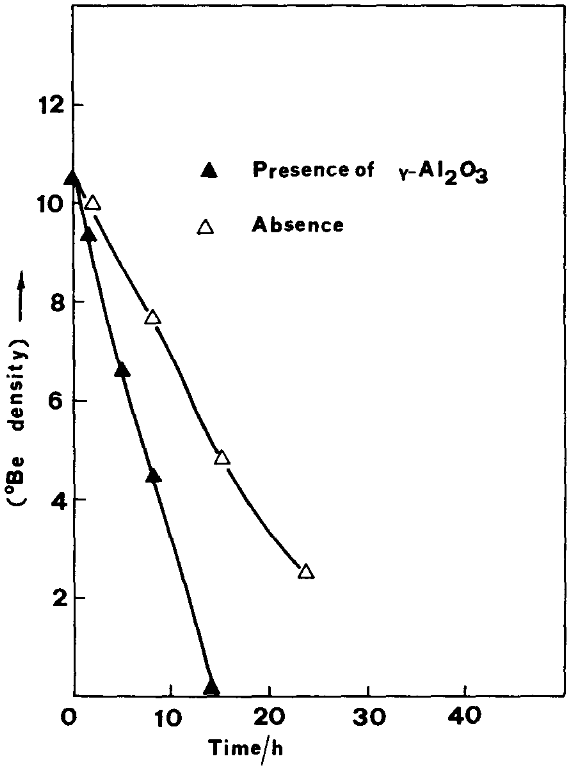

Fig. 5. Fermentation kinetics of invert-sugar observed in the presence of γ-alumina as compared to the absence of it.

the reason for the study of the influence of γ-alumina pellets on the fermentation of molasse and raisin extracts. The promotional effect of γ-alumina on the ethanol production rate and the reduction of fermentation time in the case of molasse fermentations, can be attributed to one or more of the following factors.

1. γ-Alumina promotes the activity of some of the enzymes involved in ethanol fermentation or it is an heterogeneous catalyst of the process. This seems to be correct, since γ-alumina was found to reduce the activation energy E_a of the alcohol fermentation.
2. It could be that cells immobilized on γ-alumina are more active than the free ones. According to our knowledge, the immobilization of cells on solid supports increases the ethanol production rate, but this increase is clearly lower than that obtained by the inorganic material.
3. The increase in pH caused by the influence of γ-alumina. The latter must be excluded because during fermentation, the pH was adjusted to 4.7 by sulfuric acid.

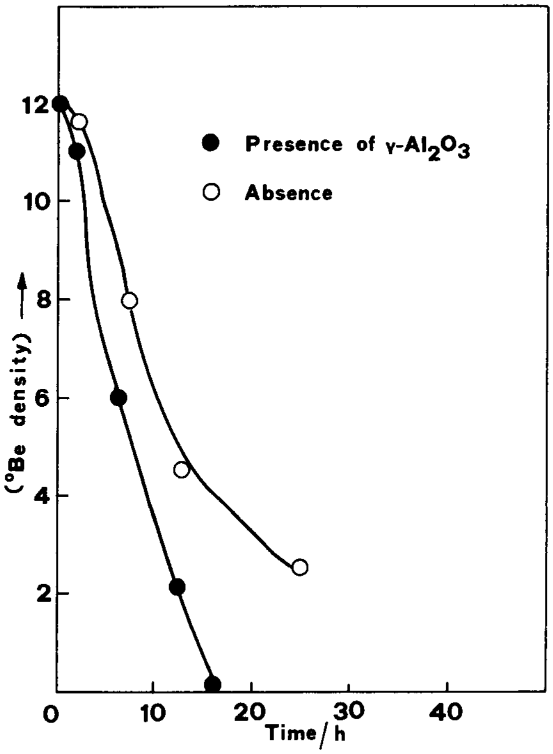

Fig. 6. Fermentation kinetics of sucrose observed in the presence of γ-alumina as compared to the absence of it.

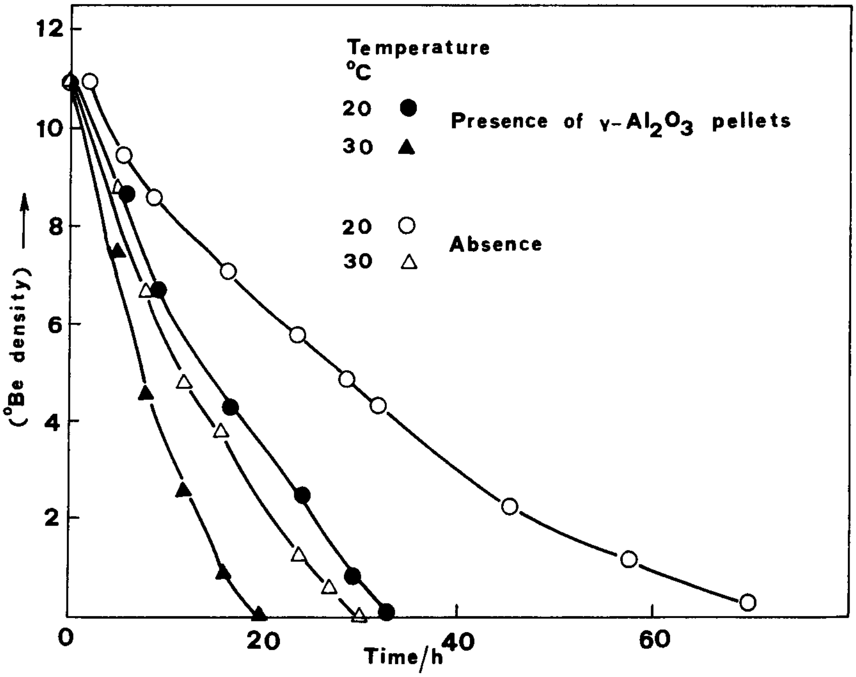

Fig. 7. Fermentation kinetics of glucose observed in the presence and in the absence of γ-alumina at temperatures of 20 and 30°C.

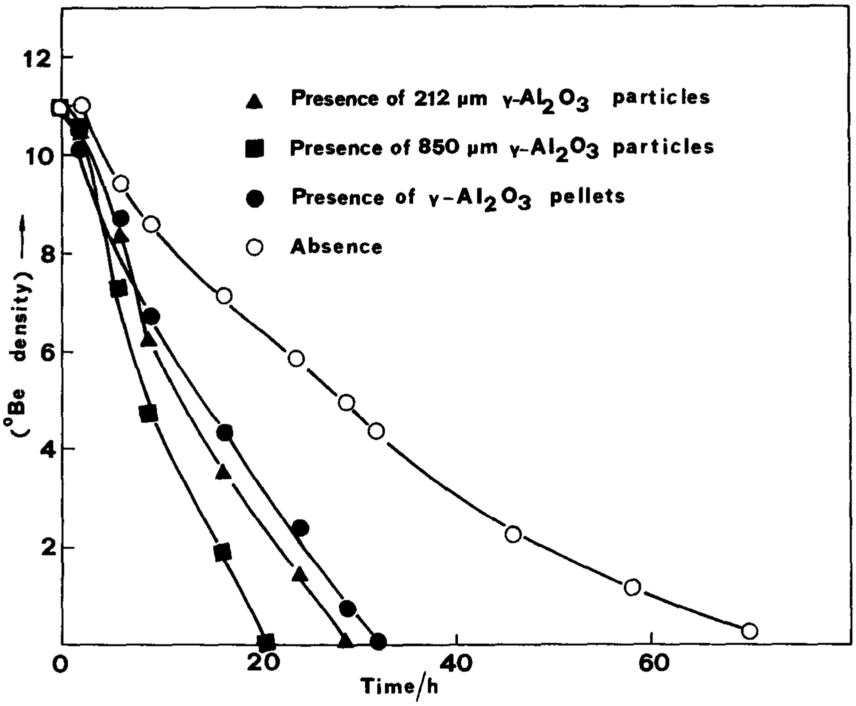

Fig. 8. Effect of γ-alumina particle size on the fermentation rate of glucose.

4. The proposed inorganic material increases the free cell concentration. Thus must also be excluded, because in molasse fermentation made in the presence of γ-alumina pellets, more cell growth than that obtained in the fermentation made with its absence has not been observed.
5. Undesirable materials contained in molasses are excluded by adsorption. This is not correct, since γ-alumina promotes the fermentation of the synthetic media containing sucrose, not that containing inhibitor materials.

The γ-alumina used in synthetic media containing glucose (2) fermentation gave analogous promotional effect to that obtained in molasse presented in this work. The positive effect of γ-alumina pellets on glucose and molasse containing sucrose makes us hope for a promotional action of γ-alumina on raisin extract, which is a raw material rich in invert sugar (glucose-fructose). The results presented in this work do not satisfy this possibility. So, γ-alumina does not promote the ethanol fermentation of raisin extracts. However, fermentations of synthetic media containing invert sugar or fructose were promoted by the presence of γ-alumina. Its promotional effect on glucose (2), fructose, and invert sugar make clear that the no promotional effect on raisin extract is not owing to the contained invert sugar. Therefore, raisin extracts contain an unidentified factor that does not properly permit γ-alumina to enhance the process.

To find this factor, we have examined pH of the fermentation medium as well as the dopping of Mg and K (coming from magnesium sulfate and dihydrogen potassium sulfate employed as nutrients in the synthetic media) on the surface of γ-alumina. The initial pH value (3.7) of raisin extracts used, is different from those (pH 5.6) used in synthetic media containing glucose fermentations. Raisin extracts fermentation in a pH adjusted to 5.6 gave similar results with that obtained in pH 3.7 value. Likewise, the addition of magnesium sulfate and dihydrogen potassium sulfate in raisin extract fermentations did not promote the process. Therefore, pH, Mg^{2+}, and K^{+} ions do not affect the promotional effect of γ-alumina. The improvement of the ethanol production rate obtained by γ-alumina in powder form stimulate the thought that the fermentation had better take place in a fluidized bed reactor. The fact that powder of 212 μm gave lower ethanol production rate than those of 850 μm, may be attributable to closing of pores by the very small particles contained.

The decrease of residual sugar followed by an accepted yield at the relatively high conversion obtained with γ-alumina, shows that fermentation takes place even at relatively high ethanol concentrations. This possibility contributes to a reduction of the energy demand of potable and grade-fuel ethanol production and diminishes the alcohol production cost. Also, the seven repeated batch fermentations process using free cells, carried out in the presence of the proposed inorganic material, made with no reduction to promoting action from batch to batch, contributes the idea of its use in the alcohol production industry without any further significant changes in the process. One may conclude that our first results show that γ-alumina does not cause any undesirable effects in the fermentation of molasse by *S. cerevisiae*. The proposed inorganic material increases markedly the ethanol production rate in the fermentation of molasse and not of rich invert-sugar raisin extract. γ-Alumina also reduces the energy demand and cost of the ethanol production employing molasse.

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