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Continuous Wine Making by Delignified Cellulosic Materials Supported Biocatalyst

An Attractive Process for Industrial Applications

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ABSTRACT

The continuous making of wine by a delignified cellulosic (DC) material-supported biocatalyst is reported. It was prepared by immobilizing the alcohol resistant strain AXAZ-1 on DC material. The product was found suitable for the continuous process in industrial applications. The operational stability was maintained for 2 mo with monitoring the ethanol concentration, wine, and alcohol productivities as well as the stability of °Be density at the outlet. Wine productivity was three- to sixfold higher than obtained by natural fermentation, alcohol concentrations of the wine was in the range of 9.3–11.2% v/v and low volatile acidities of 0.15–0.36 g acetic acid/L were obtained. The effect of total acidity and flow rate of must were also examined. To demonstrate that the operational stability of the bioreactor is due to DC material that promotes the fermentation, and it takes place at even higher ethanol levels, an analogous system of kissiris supported biocatalyst was studied. Likewise, the tolerance in the alcohol concentration, as compared with free cells, were studied by their stability of the activity in the repeated batch fermentation of must.

Index Entries: Delignified cellulosic, continuous; wine making; operational stability; alcohol resistance.

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INTRODUCTION

Continuous wine making and alcohol production increases the productivity and reduces production costs. Therefore, cell immobilization has drawn considerable attention in recent years and publications have appeared discussing continuous wine making by immobilized cells. More specifically, grape wine was continuously produced by fermentation of grape juice with immobilized cells in alginates (*1–3*). A method for continuous production of sparkling wines by yeast immobilized on a sorbent was patented (*4*). Likewise, the product DEAE-cellulose (*5*) covered by anion exchange resin was used. Mineral Kissiris was employed for yeast immobilization to obtain continuous low temperature wine making (*6*). However, grape wine is not yet produced by continuous fermentation in industry, because of prerequisites concern for food-grade purity of the support, taste, and aroma of the wine and cost-effective immobilization. The last factor needs: high viability of immobilized cells and, therefore, high operational stability of the bioreactor, availability of a low cost support material, and abundance and accessibility of the material. As a result of relatively high alcohols content of wines (11–12% v/v), a high operational stability needs an alcohol resistant system of immobilized cells on the solid support. This is a disadvantage since the yeast cells are not resistant enough in alcohol at the above concentration for the time needed for continuous fermentation. The problem of high alcohol content, there is no incontinuous potable alcohol production, where 7–8% v/v ethanol concentrations are required.

Recently, immobilization of yeast on DC material (*7*) was obtained. It was found that this biocatalyst prepared decreases the activation energy Ea, increases the rate of the fermentation, and makes the fermentation taking place at even higher ethanol concentration (*8*). Furthermore, the alcohol-resistant strain AXAZ-1 of *Saccharomyces cerevisiae* species, from the Greek agricultural area, was isolated (*9*). Therefore, the immobilization of the yeast strain AXAZ-1 on DC material may be lead to a suitable biocatalyst for continuous wine making with industrial applications. This possibility was the aim of the present investigation.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

The alcohol-resistant strain of AXAZ-1 of *Saccharomyces cerevisiae* species was employed. It was grown in a liquid medium contianing 2% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.5% $MgSO_4 \cdot 7H_2O$ in distilled water. Pressed wet weight cells (20–25 g) were produced as follows: Strain AXAZ-1 inocula were prepared in a 5000 mL culture at late log phase, pH 5.6. The culture was incubated without

agitation in 30°C until late log cells were separated by centrifugation. They were transfered in the reactor for the start up of the process. The continuous culture media in the case of glucose solutions contained 113 g/L glucose and the other nutrients as above described. All media were sterilized at 130°C for 15–20 min. Reactor temperature was controlled at 27°C by placing the reactor in a constant temperature water bath. For the cell growth the pH was adjusted to 5.6.

Preparation of Grape Must

Grape must were prepared from cultivar kerino. In order to adjust total acidity to 6, 8, and 10 g tartaric acid/L, samples were titrated with 0.1N NaOH to determine total acidity of must and then, additionally, tartaric acid was added. All must was used without nutrient addition and sterilized at 130°C for 15–20 min. The initial °Be density was adjusted to 12 °Be, by the addition of sucrose or water in the case which must was more concentrated.

Preparation of DC Material

It was prepared after lignin removal from sawdust according to a previous method (*7*). The DC was a wet and frothy solid support.

Analytical Procedures

Ethanol was determined as alcoholic degrees (mL ethanol/100 mL wine) obtained after distillation of samples using a Gay Lusacc alcohol meter.

Residual sugar was determined in all samples by the Lane-Eynon method (*10*).

Volatile acidity was determined (*11*) after steam distillation of samples and titration of the distillate with 0.1 NaOH.

Repeated Batch Fermentations

The strain AXAZ-1 was immobilized on DC material and repeated batch fermentations of must was performed as previously described (*7*). At the same time, batch fermentations were also performed using must containing similar concentration of free cells as compared with immobilized. It was found that the cells immobilized on DC material were 4.9 g wet weight per 100 g of DC material (*7*). Fermentations were carried out in a pair of cylinders, one containing DC material-supported biocatalyst, and the other separately free cells. Musts were prepared with an initial °Be density of 4.4 and initial alcohol concentration 10% and fermentations were performed at 25°C. Free cells were used from the first to second batch obtaining the same cell concentrations after centrifugation of the liquid of first fermentation. DC material supported biocatalyst was used from

batch to batch after its washing one time with must. Kinetics of the fermentations were also performed to each sample of every pair by measuring the °Be density at various time intervals.

Pilot Plant

Continuous wine making was performed in a continuous reactor system. It was a glass tower reactor (1400 mL total working volume) containing 500 g of the DC material placed in the reactor as the immobilization support for the continuous process. The upper exit of the bioreactor was blocked with glasswool in order to prevent passing of the DC material. Must was pumped in an upflow stream with the aid of a high accuracy peristaltic pump (Cole Parmer Intrument Co., Chicago, IL).

Experimental Procedure

The reactor was charged with culture media containing 113 g/L glucose and 20 g/L wet-weight cells of the *Saccharomyces cerevisiae* strain AXAZ-1, to cover all the support material. The pH was adjusted to 4.7 and the mixed culture was allowed to ferment without feeding. After 4 h the synthetic media containing 113 g/L glucose and having a pH 5.6 was pumped in a flow rate of 1000 mL/d. The fermentor was operated continuously for 120 h for biomass attachment. Subsequently, the reactor was supplied with grape must for 58 d with a flow rate in the range of 470–1300 mL/d. The initial °Be density remained relatively constant.

The effect of total acidity of must and the flow rate in the reactor on its operational stability were studied. The operational stability of the reactor was examined by measuring every day the °Be density of the influent and efluent as related to pH and flow rate of must. Likewise, the operational stability was also examined by the determination of alcohol concentration, residual sugar, total acidity, calculation of wine and alcohol productivities as related with the total acidity of must, and flow rate of the reactor.

Samples of the effluent were collected every 24 h when steady state was obtained and analyzed for °Be density, residual sugar, alcohol concentration, and volatile acidity.

Wine productivity was expressed as Kg wine/L of the reactor volulme produced in 1 d and calculated by dividing the Kg of wine produced per day by the liters of the total working volume of the reactor. Ethanol productivity, given on Table 1, was expressed as grams of ethanol per liter in one day and calculated on the basis of total working volume, by multiplying the dilution rate by ethanol concentration.

Demonstration of the Operational Stability of the Bioreactor by an Analogous System

In order to prove that the important operational stability of the bioreactor can be attributed in the even higher ethanol concentrations obtained

Table 1
Wine Characteristics in the Continuous Wine Making by Delignified Cellulosic Materials Supported Biocatalyst as Related to Total Acidity of Must and Volumetric Rate

Flow rate of the reactor, mL/d	Duration, h	°Be density of the influent	Alcohol conc., % v/v	Residual sugar, g/L	Wine productivity, Kg/L·d	Ethanol productivity, g/L·d	Total acidity of must, g tartaric acid/L	Volatile acidity, g acetic acid/L
600	24	12.1	10.6	39.6	0.43	37	6	0.16
500	24	12.0	10.6	28.3	0.36	31	6	0.15
500	24	12.0	10.8	26.7	0.36	31	6	0.17
480	24	12.0	10.8	28.3	0.35	30	6	0.15
480	24	12.0	10.9	25.4	0.35	30	6	0.15
500	24	12.1	10.7	29.3	0.36	31	6	0.15
490	24	12.0	10.6	38.2	0.35	31	6	0.15
490	24	12.0	10.8	39.1	0.35	30	6	0.16
470	24	12.0	10.7	41.2	0.34	29	6	0.15
480	24	12.0	10.9	37.1	0.35	30	6	0.15
500	24	12.1	10.8	29.3	0.36	31	8	0.26
510	24	12.2	11.1	25.1	0.37	33	8	0.24
520	24	12.1	10.9	—	0.37	—	8	—
510	24	12.1	11.2	29.3	0.37	33	8	0.24
520	24	12.2	10.8	30.5	0.37	32	8	0.24
520	24	12.2	10.8	28.5	0.37	32	8	0.24
580	24	12.0	9.3	30.2	0.42	31	10	0.29
580	24	12.0	9.4	24.0	0.43	32	10	0.31
580	24	12.0	9.3	19.6	0.43	32	10	0.33
580	24	12.0	9.3	22.6	0.41	31	10	0.32
580	24	12.0	9.3	25.6	0.41	32	10	0.34
580	24	12.0	9.4	27.8	0.41	31	10	0.32
800	24	12.0	10.8	26.3	0.58	59	6	—
1300	24	12.0	9.7	49.7	0.94	88	6	—
1100	24	12.0	9.8	52.3	0.79	62	6	0.36
800	24	12.0	10.8	24.7	0.58	50	6	0.32
800	24	12.0	9.8	32.6	0.58	45	6	0.33
800	24	12.0	9.9	29.3	0.58	45	6	0.30

by DC material, similar experimental procedure was also performed using mineral kissiris which have similar effect (*6,12*).

Continuous wine making was performed in the same pilot plant following about the same procedure. The reactor was charged with 700 g mineral kissiris and the immobilization was made according to a previous study (*13*), using the strain AXAZ-1.

Likewise, repeated batch fermentations were made according to the procedure described for DC material, with differentiation that the immobilized cells on mineral kissiris was 6 g wet weight cells per 150 g of the mineral (*6*).

RESULTS AND DISCUSSION

Operational Stability of the Bioreactor

In order to examine the DC material-supported biocatalyst for its convenience in industrial continuous wine making, we have studied the operational stability of bioreactor as related to total acidity and flow rate of must. The system was pumped with must for about two months with relatively constant initial °Be density. The flow rate was relatively constant and the acidity of the must was successively increased after the addition of the appropriate amount of tartaric acid. Likewise, to study the effect of the flow rate of must, the acidity remained constant and the reactor was pumped in a flow rate in the range of 800–1300 mL/d as it compared to those of 400–600 mL/d. The results are summarized in Table 1 and Fig. 1.

One can observe (Table 1) for a long period, alcohol concentration in the range of 9.3–11.2% v/v as well as constant wine and alcohol productivities. The alcohol concentration dropped when the total acidity of must was increased to 10 g tartaric acid/L. An inspection of the Fig. 1 also shows that the operational stability of the reactor was constant as indicated in the effluent by the stability of °Be density. The alcohol concentration was higher than 10% v/v and the operational stability of the reactor was not affected as the total acidity increased from 6 to 8 tartaric acid/L. The system can operate up to a wine productivity of 0.60 Kg/L·d without any significant reduction in alcohol concentration. Likewise, volatile acidity was at a very low level, actually lower than those of wines produced by free cells. Volatile acidity was not significantly affected by the change of total acidity and flow rate.

The above presentation of results indicate that DC material-supported biocatalyst can be used for wine making by continuous fermentation with excellent operational stability of the reactor. The later can be supported by the stability for a long period at accepted levels of alcohol concentration, volatile acidity, and wine productivity.

Wine and alcohol productivities were about sixfold higher than those obtained by natural fermentation. Maintaining operational stability of the

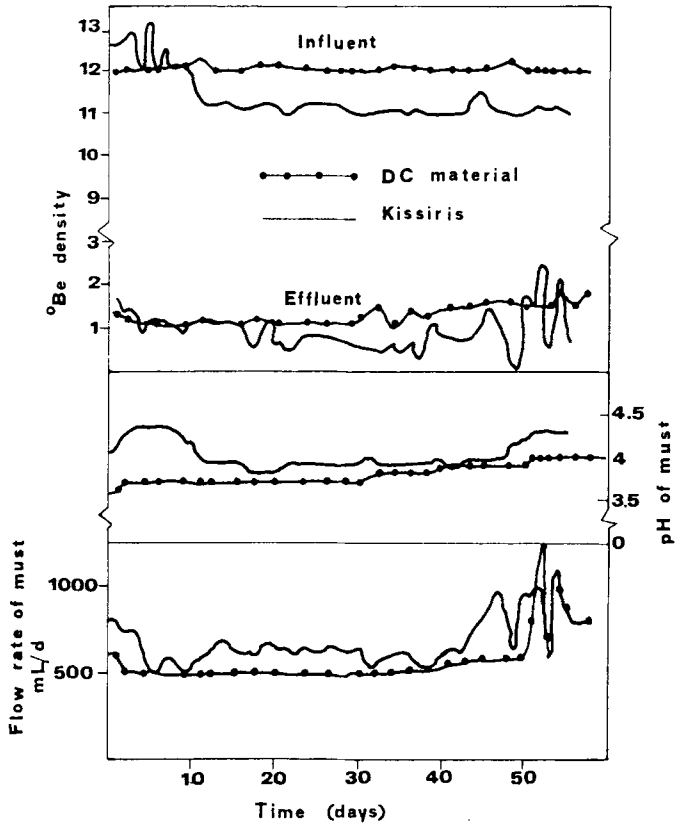

Fig. 1. Operational stability of the reactor relative with °Be density of the effluent as related with pH and flow rate of must.

reactor at 6–10 g tartaric acid/L total acidity, allows the process to be employed in southern countries producing musts of low total acidities as well as in the north where wine musts contain relatively high concentrations of organic acids. Likewise, the operational stability of the reactor over a long period, in conjunction with its low cost and abundance of the support material, give an advantage to the process for its industrial application.

Demonstration of the Operational Stability by an Analogous System

Recent papers report that DC material-supported biocatalyst (*7,8*) and kissiris-supported biocatalyst (*6,12*) reduce the activation energy Ea, increase the ethanol production rate, and allow the fermentation to take place at even higher ethanol concentrations than free cells. In order to determine the greater operational stability of the reactor using DC material, may be attributed to the above effects, continuous wine making by a kissiris supported biocatalyst was also studied. The results are summarized in Table 2 and Fig. 1. These results clearly show that a kissiris-supported

Table 2
Wine Characteristics in the Continuous Wine Making by Kissiris-Supported Biocatalyst as Related to Total Acidity of Must and Volumetric Rate

Flow rate of the reactor, mL/d	Duration, h	°Be density of the influent	Alcohol conc., % v/v	Residual sugar, g/L	Wine productivity, Kg/L·d	Ethanol productivity, g/L·d	Total acidity of must, g tartaric acid/L	Volatile acidity, g acetic acid/L
700	24	11.2	10.0	21.8	0.50	40	4	0.13
620	24	11.2	10.0	21.3	0.43	35	4	0.14
600	24	11.0	10.1	20.2	0.42	35	4	0.12
620	24	11.1	11.2	12.1	0.43	40	4	0.20
620	24	11.1	11.2	12.3	0.43	40	4	0.22
700	24	11.1	11.3	11.8	0.50	45	4	0.19
650	24	11.2	11.5	13.7	0.46	43	4	0.22
650	24	10.9	11.3	12.5	0.45	42	4	0.25
550	24	10.9	11.5	11.5	0.39	36	4	0.27
600	24	11.2	10.8	24.1	0.42	37	4	0.34
500	24	11.0	10.2	22.3	0.35	29	6	0.30
550	24	11.0	10.2	23.1	0.39	31	6	0.28
600	24	11.0	10.1	23.3	0.42	34	6	0.29
600	24	11.0	10.0	22.9	0.42	34	6	0.32
610	24	11.0	10.1	20.5	0.43	35	6	0.30
620	24	11.1	10.0	21.1	0.43	35	6	0.31
500	24	11.1	10.0	20.7	0.35	29	8	0.30
500	24	11.0	10.1	21.1	0.35	29	8	0.32
600	24	11.1	10.1	23.5	0.42	35	8	0.35
650	24	11.0	10.0	23.3	0.46	37	8	0.30
650	24	11.0	10.0	19.6	0.46	37	8	0.32
600	24	11.0	10.1	21.5	0.42	34	8	0.36
1000	24	11.5	9.2	35.8	0.70	53	4	0.25
950	24	11.0	9.2	33.5	0.67	50	4	0.22
950	24	11.0	9.4	38.0	0.67	51	4	0.19
1000	24	11.0	9.3	42.0	0.70	53	4	0.23
1050	24	11.1	8.7	54.0	0.74	52	4	0.24
850	24	11.1	9.9	35.0	0.60	48	4	0.24

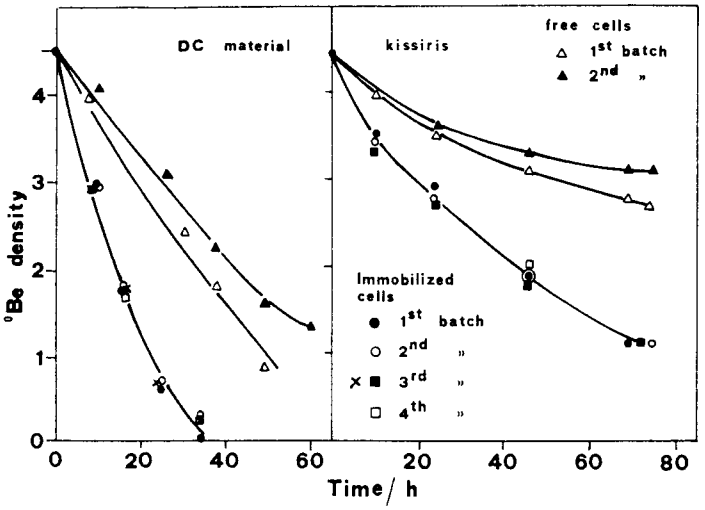

Fig. 2. Comparison at kinetics of DC material- and kissiris-supported biocatalysts with free cells used in the fermentation of must with initial °Be density of 4.4 °Be and initial alcohol content of 10%.

biocatalyst produces wine for a long period with an alcohol concentration in the range of 10–11.5% v/v at total must acidities of 4–8 g tartaric acid/L. Likewise, wine and alcohol productivities were 0.35–0.74 Kg/L·d and 29–53 g/L·d, respectively. An inspection of Fig. 1 shows that for the same period the drop of °Be density from the influent to effluent was similar to that obtained with the DC material. Therefore, the presentation of results shows that the operational stability of the reactor is about similar with that obtained by DC material, and the above thought may be confirmed.

The Alcohol Resistance of Biocatalysts

To prove that the large operational stability of the reactor is because of the fact that DC material and kissiris-supported biocatalysts are more alcohol resistant than free cells, the strain AXAZ-1 was immobilized on DC material and kissiris. Thus, the biocatalysts prepared were compared in repeated batch fermentations with free cells. The results are presented in Fig. 2.

In order to obtain similar conditions with continuous fermentation, we used high initial alcohol concentration and relatively low initial °Be density. In order to proceed from batch to batch, DC and kissiris-supported biocatalysts were washed once with must, but separately free cells were centrifutaged. In the case of free cells the appropriate cell mass was transferred, in must, to obtain the same cell concentration as with the immobilized cells.

Fig. 2 shows that the immobilized cells on DC material and mineral kissiris ferment faster than the free cells. Likewise, in this figure one can

observe: the fermentation of free cells was stopped at higher °Be density than with immobilized cells. Using free cells, the final °Be density of the second batch was higher than the first. The alcohol production rates were not reduced from batch to batch in the repeated batch fermentations performed with DC material- and kissiris-supported biocatalyst.

The operational stability of the reactor for a long period indicates better vitality of immobilized cells on DC material and mineral kissiris than with free cells. The later is supported by the results of Fig. 2 where higher alcohol production rates and lower final °Be densities were obtained with immobilized cells. Likewise, no increase in the fermentation time and final °Be density achieved by DC material- and kissiris-supported biocatalysts as compared with free cells in the repeated batch fermentations. This may be attributed to the fact that the strain AXAZ-1 is alcohol resistant (*9*), and DC material as well as mineral kissiris give even higher final ethanol concentrations than free cells (*8,12*).

CONCLUSIONS

DC material-supported biocatalyst prepared by immobilization of the alcohol resistant strain AXAZ-1 on DC material is suitable for continuous wine making. This is supported to the operational stability of the bioreactor obtained, as a result of the alcohol resistance of the prepared biocatalyst. The proposed biocatalyst is an attractive material for industrial application of continuous wine making. The latter can be supported to the fact that DC material can be in the future abundant for all countries, having low cost and a solid which does not release any contaminants in the wine. Also, it gives sixfold higher ethanol productivity than the natural fermentation. Alcohol-resistant solid supported biocatalysts can be prepared by the immobilization of an alcohol resistant strain on supports, promote the fermentation and make it take place at even higher ethanol concentration than free cells.

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