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Production of alcohol from sugar beet molasses without heat or filter sterilization

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Among three esters of *p*-hydroxybenzoate, n-butyl *p*-hydroxybenzoate was selected as the best antimicrobial substance. Molasses medium sterilized by this ester was used as a substrate for ethanol production. n-Butyl *p*-hydroxybenzoate (0.15% w/v) completely inhibited the growth of free yeast cell inoculum, Ca-alginate immobilized yeast inoculum and bacterial contaminants. Immobilization of the yeast cell inoculum in Ca-alginate with castor oil (6% v/v) offered a yeast cell protection against the inhibitory effect of n-butyl *p*-hydroxybenzoate. The presence of castor oil in this immobilization system did not affect the metabolic activity of the yeast in beads compared to the cells immobilized without castor oil. The yeast cell beads in this system completely utilized up to 25% molasses sugar with an ethanol yield of 10.58%, equal to 83% of its theoretical value. The beads were stable and could be used successfully for seven cycles of batch fermentation. The optimum fermentation temperature using this system was 35°C.

Keywords: molasses; n-butyl *p*-hydroxybenzoate; castor oil; co-immobilized cells; reutilization

Introduction

Since the cost of raw materials make up 55–75% of the final alcohol selling price, alcohol production from low-priced materials has become an important area of investigation. Molasses, a by-product of the sugar industry, represents a ready and renewable source for ethanol fermentation because of its high availability and low cost. Molasses may vary somewhat in composition but usually contains about 50–55% fermentable sugar. For economic [2] and scientific [1] reasons, some techniques have been reported for microbial fermentation without heat or filter sterilization. The use of high initial sugar concentration and low initial pH [8] and the addition of specific substances [3], that are inhibitory to contaminant microorganisms but to which the culture inoculum is resistant, have been exploited. However these methods are applicable only when the fermentation microorganism has some special characteristics. Therefore a general method of fermentation without pre-heating or filter sterilization of both media and fermentor is required. Two reports illustrate how cells can grow normally without pre-sterilization. Tanaka *et al* [6] indicated that cells immobilized in Ca-alginate with vegetable oils can grow in a medium initially containing a toxic solvent (2-octanol, benzene, toluene or phenol) without first removing the solvent. Also Tanaka *et al* [7] indicated that antimicrobial chemicals used for food preservation such as the esters of *p*-hydroxybenzoic acid, can be used for sterilization of fermentation media. They found that immobilization of the fermentation microorganism in alginate with vegetable oil has a protective function against these substances. No more work has been done to confirm this new technique. This current study develops this technique in the fermentation of lower-cost materials such as the industrial or agricultural wastes which are frequently contaminated with antimicrobial substances toxic to microbial cells. The model is a system of ethanol production from sugar beet molasses and optimizes the effect of essential parameters eg temperature, re-utilization of beads and sugar concentration on stability and activity of oil-Ca-alginate beads.

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Materials and methods

Materials

Sugar beet molasses was obtained from the Sugar factory, Kafer El-shakh, Egypt and contained 50% fermentable sugar. Sodium alginate, n-butyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate and methyl *p*-hydroxybenzoate were obtained from Sigma Co, Regensburg, Germany. High quality castor oil and chemicals were obtained from commercial sources, Cairo, Egypt.

Organism

Saccharomyces cerevisiae NCYC 975 was obtained from the National Collection of Yeast Cultures, Norwich, UK. It was maintained on YM agar medium which consisted of yeast extract, 3 g; malt extract, 3 g; peptone, 5 g; glucose, 10 g; agar, 20 g; all dissoved in 1 L of distilled water and adjusted to pH 5.6. The medium used to grow cells for a free cell inoculum and for cell immobilization contained per liter of distilled water: molasses, 200 g (10% sugar); urea, 1.08 g; $MgSO_4$, 0.3 g; and phosphoric acid, 0.3 ml. The pH was adjusted to 4.6 with 0.5 M citric acid and autoclaved for 15 min at 120°C. An inoculum was prepared by washing a yeast slope with 2 ml sterile Ringers solution and added to 100 ml of the above medium. After 30 h cultivation at 30°C the culture contained approximately 5×10^8 cells per ml.

Immobilization

Eighty-four millilitres of sterile sodium alginate (3% w/v) were mixed with 10 ml pre-cultured yeast suspension and

6 ml of castor oil. The mixture was emulsified by stirring at 2000 rpm for 12 min and then extruded through a sterile Pasteur pipette into 200 ml of 2% $CaCl_2$ sterile solution. The resulting beads (approximately 2 mm in diameter) were cured for 1 h at room temperature before use. For immobilization without castor oil, 90 ml of 2.8% (w/v) sterile sodium alginate and 10 ml pre-cultured yeast cells were used.

Determination of lethal dose of each ester

Seven concentrations of each ester (0, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3% w/v) were used to determine their lethal dose (100 ml medium per Erlenmeyer flask) with the free cell inoculum and bacterial contaminants. The flasks were inoculated with 1.5 ml of the yeast cell suspension. Three replicates for each treatment were carried out and the flasks were kept in a shaking incubator at 30°C. After 48 h of incubation the viable yeast cells and total count of bacteria were enumerated.

Activity of free-cells, immobilized cells and co-immobilized cells with castor oil

Molasses medium consisting of molasses, 300 g (15% sugar); urea, 1 g; $MgSO_4$, 1 g and phosphoric acid, 0.3 ml was sterilized by autoclaving it for 15 min at 120°C. Erlenmeyer flasks (100 ml) were divided into three groups (three replicates). The first group was inoculated with 1.5 ml of the yeast cell suspension, the second group with 12 g of cell beads with castor oil (equivalent of cells in the 1.5-ml cell suspension) and the third group was inoculated with 12 g of beads without castor oil. The cultures were incubated in a shaking incubator at 30°C. Residual sugar was determined every 6 h.

Activity of immobilized cells and co-immobilized cells with castor oil in the presence of n-butyl p-hydroxybenzoate

Molasses medium (100 ml/Erlenmeyer flask) with pH 5 was used to compare the growth of immobilized cells and co-immobilized cells with castor oil in the presence of n-butyl *p*-hydroxybenzoate (0.15%) as a sterilizing agent. There were two groups (three replicates) of flasks: (a) inoculated with 12 g of beads with castor oil; and (b) with 12 g of beads without castor oil. Consumption of molasses sugar was considered as an indicator for the cells' growth and activity.

Batch fermentation

The fermentation medium contained a range of molasses according to the desired sugar concentration. The fermentation was carried out in a 100-ml column (22.5×2.5 cm) loaded with 54 g of beads. Medium was recycled from a holding vessel (450 ml) at 20 ml min^{-1} (peristaltic pump Mastergflex, Cole Parmer Inc, New York, USA). A fermentation lock allowed the escape of CO_2 (Figure 1). The temperature was regulated and samples were periodically withdrawn from the medium vessel for analysis.

Re-utilization of beads

The assay was started as indicated in batch mode. Every 18 h the molasses medium was drained off and replaced by an equal amount of fresh medium sterilized by adding 0.15% n-butyl *p*-hydroxybenzoate. Ethanol and residual sugar were determined.

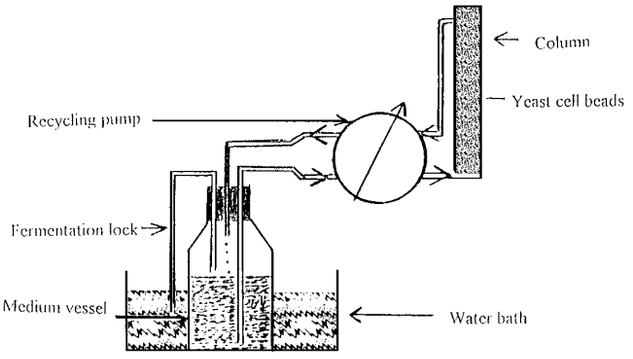

Figure 1 Scheme of the experimental apparatus for ethanol fermentation of sugar beet molasses using co-immobilized yeast cells with castor oil.

Cell enumeration

Yeast cell populations were enumerated by pour plate counts on agar medium with the same composition as culture maintenance medium. Total count of bacteria was estimated by pour plate counts on nutrient agar medium which consisted of beef extract, 3 g; peptone, 10 g; agar, 20 g; all dissolved in 1 L distilled water and adjusted to pH 7.2.

Analysis

Ethanol was determined using a Hewlett Packard 5111 gas chromatograph with a flame ionization detector and a 2-M stainless steel column with SP-1500 packing. The column temperature was programmed between 60 and 190°C. Detector temperature was 250°C and injected port temperature was 200°C. Residual sugar concentration was determined by the Phenol sulfuric acid method [4].

Results and discussion

The lethal dose of each ester to the yeast cells was first determined (Figure 2). Esters were inhibitory. n-Butyl *p*-

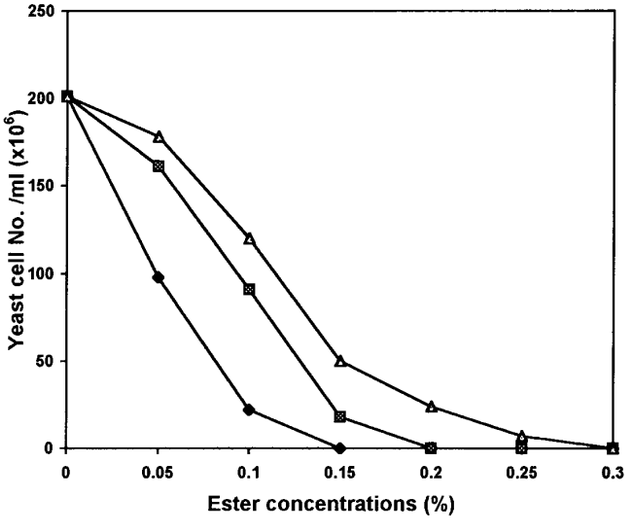

Figure 2 The doses of n-butyl *p*-hydroxybenzoate (–◆–), ethyl *p*-hydroxybenzoate (–▨–) and methyl *p*-hydroxybenzoate (–Δ–) lethal to *S. cerevisiae* cells.

hydroxybenzoate was the most efficient antimicrobial substance and completely inhibited the growth of *S. cerevisiae* in molasses medium at 0.15% whereas 0.20% and 0.30% of ethyl *p*-hydroxybenzoate and methyl *p*-hydroxybenzoate respectively were needed to completely inhibit the yeast growth. The dose of each ester lethal to the yeast cells was also lethal to bacterial contaminants. The antimicrobial activity of the esters of benzoic acid was correlated with the length of the alkyl group (Figure 2). The longer the length of the alkyl group, the more the inhibitory effect on the cells [7]. n-Butyl *p*-hydroxybenzoate (0.15%) was selected as a sterilizing agent in the subsequent experiments.

In a heat-sterilized molasses medium (no ester added), free cells completely utilized the molasses sugar after 48 h of growth (Table 1). The immobilized yeast cells utilized sugar (150 g L^{-1}) completely in only 18 h of growth. This confirms the previous findings of other workers who reported very high efficiency of immobilized yeast and bacterial cells as compared to free cells [9,10]. The results with the cells co-immobilized with castor oil showed the same activity of the immobilized cells used in 18 h. This is in agreement with the findings of Tanaka *et al* [7].

Figure 3 shows that the presence of n-butyl *p*-hydroxybenzoate (0.15%) has no effect on the metabolic activity of co-immobilized cells with castor oil. The cells completely utilized the present sugar (150 g L^{-1}) after 18 h of growth indicating that this immobilization system with castor oil offered a high protection against the antimicrobial substance. This is due to the fact that when castor oil was co-immobilized with yeast cells, the cells grew only in the water/gel phase of the gel beads (as shown by microscopic examination of bead cuts) where the water-soluble substrates such as sugar were retained. On the other hand the n-butyl *p*-hydroxybenzoate that was diffused into the gel beads was retained in the oil phase of the beads [7]. Consequently the immobilized cells found in the water phase of the beads were safe from the inhibitory effect of the used ester. The results presented in Figure 3 revealed that the consumption of molasses sugar by the immobilized cells without castor oil was greatly affected by the presence of n-butyl *p*-hydroxybenzoate and the metabolic activity of cells completely inhibited after 6 h of growth.

Table 1 Consumption of molasses sugar by *S. cerevisiae* cells in different forms

Time (h)	Residual sugar (g L^{-1})		
	Free cells	Immobilized cells	Co-immobilized cells with oil
0	150	150	150
6	141	94	97
12	122	23	25
18	108	0	0
24	90	0	0
30	62	0	0
36	41	0	0
42	19	0	0
48	0	0	0

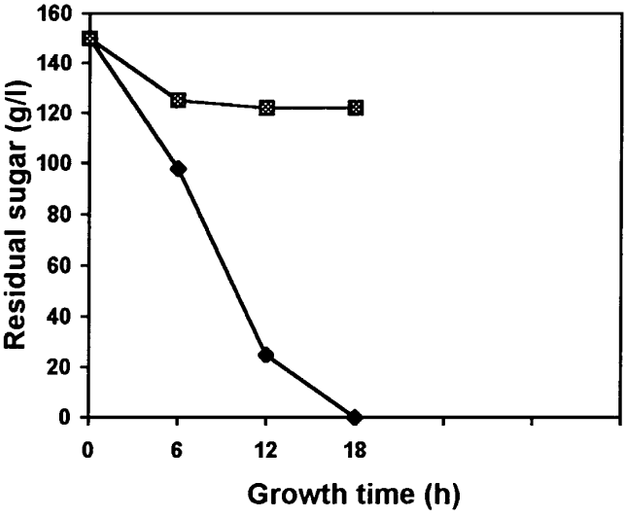

Figure 3 Consumption of molasses sugar by *S. cerevisiae* immobilized in a Ca-alginate with (–◆–) or without (–▨–) castor oil in the presence of n-butyl *p*-hydroxybenzoate (0.15% w/v).

The progress curve of fermentation of sugar beet molasses shown in Figure 4 indicated that the co-immobilized cells with castor oil retained a very high metabolic activity. A maximum ethanol yield of 6.88% (equal to 90% of its theoretical value) was achieved after only 18 h of fermentation with a complete utilization of the molasses sugar. Prolonging fermentation from 18 to 24 h reduced ethanol yield to 4% indicating that ethanol utilization occurred in the later stages of fermentation as observed by other authors [9].

The yeast entrapped in Ca-alginate with castor oil showed no loss of activity after seven cycles in batch mode with100% utilization of the sugar and ethanol yield equal to 90% of its theoretical value. The beads were then separated from molasses medium, placed in fresh medium (sterilized with n-butyl *p*-hydroxybenzoate) and stored at 4°C. After 15 days, the fermentation assays were reassumed

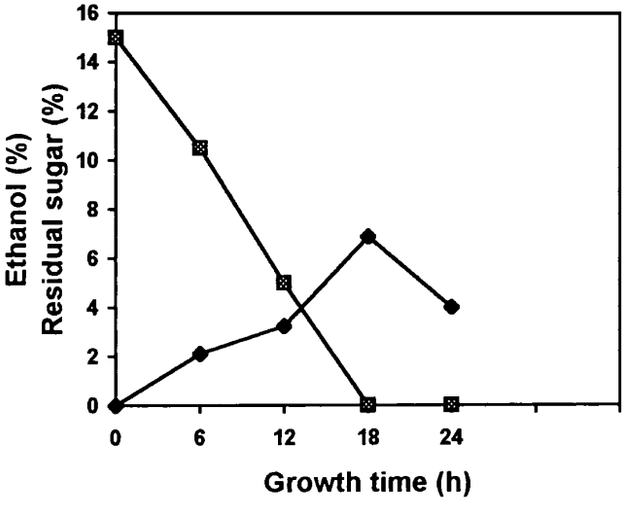

Figure 4 Percentage of residual sugar (–▨–) and ethanol (–◆–) yield from molasses medium sterilized by n-butyl *p*-hydroxybenzoate using *S. cerevisiae* immobilized in Ca-alginate with castor oil.

Table 2 Effect of sugar concentrations on ethanol yield by *S. cerevisiae* immobilized in Ca-alginate with castor oil

Sugar conc. (%)	Ethanol yield (%)	Residual sugar (%)	Running time (h)	Theoretical value of ethanol yield (%)
15	6.88	0	18	90
20	9.18	0	30	90
25	10.58	0	42	83
30	7.72	11	54	50
35	0	35	72	0

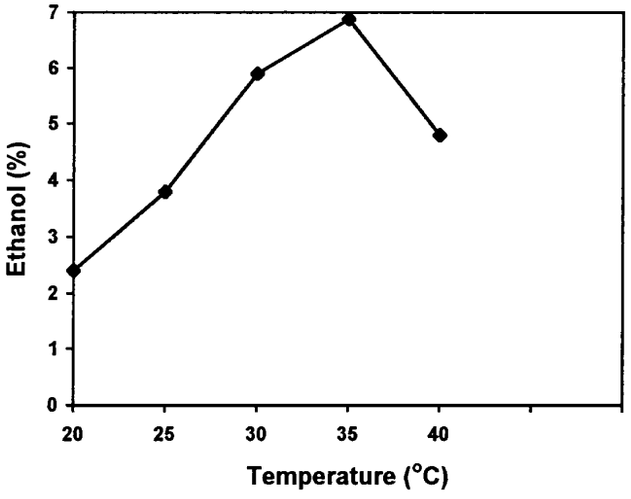

Figure 5 Effect of fermentation temperature on ethanol yield from molasses medium sterilized by n-butyl *p*-hydroxybenzoate using *S. Cerevisiae* immobilized in Ca-alginate with castor oil.

and the beads produced the previous levels of ethanol with complete utilization of sugar. The beads were stable and showed no apparent changes in structure. However fissures in some beads were observed at the end of the experiment. This may be due to the generation of CO_2 inside them. However these fissures did not affect the metabolic activity of the beads.

Sugar concentration up to 15% was found to be inhibitory to fermentation processes using free yeast cells although the exact figure is strain-dependent [5]. Therefore it was important to study the ability of co-immobilized yeast with castor oil to ferment high molasses sugar concentration into ethanol. Table 2 shows that the used strain in this system was able to utilize 25% sugar completely, after 42 h of fermentation with an ethanol yield of 10.58%, equal to 83% of its theoretical value. At concentrations above 25% sugar, it was found that the ethanol yield started to decline due to osmotic phenomena in which plasmolysis of the yeast cells begins to occur. These results indicate that the yeast in this immobilization system showed good tolerance to high sugar and alcohol concentration in the medium and gave rapid fermentation with no residual sugar.

The results in Figure 5 show that, not unexpectedly, increasing the fermentation temperature from 20 to 35°C resulted in a three-fold increase in ethanol yield. The ethanol yield decreased when the temperature was raised to 40°C.

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