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Production of mead by immobilized cells of *Hansenula anomala*

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Summary. Mead was produced by immobilized cells of *Hansenula anomala* in calcium alginate gels. The immobilized cell beads of 3 mm diameter packed in column reactors of dimensions 2.2×60, 4×40 and 8×80 cm, produced mead containing maximum concentrations of ethanol and ethyl acetate of 70 g/l and 730 mg/l, respectively at a dilution rate of 0.1 h^{-1}. The maximum alcohol productivity achieved was 23.1 g/l·h at a dilution rate of 0.33 h^{-1}. With intermittent regenerations of the cells the reactor operated continuously for 110 days. This process enables the quick production of matured mead by a single culture and the elimination of the traditionally used long aging periods.

Introduction

Traditional production of meads have been reported by Jorczyk and Wzorek (1977) followed by a long aging process for their maturation. Qureshi and Tamhane (1985) produced meads by immobilized cells of *Saccharomyces cerevisiae* and considerably reduced time of fermentation but not of the maturation. Further Qureshi and Tamhane (1986) produced matured meads using immobilized cells of *S. cerevisiae* BRL-7 producing ethyl alcohol and *Hansenula anomala* producing ethyl acetate packed in two series reactors. The meads thus produced required no aging.

In view of the economic feasibility, the immobilized cells of *H. anomala* were studied to produce matured meads, thus avoiding dual culture fermentation and double series reactors. The results obtained are presented in this paper.

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Materials and methods

Organism. An osmophilic yeast strain of *H. anomala* which was isolated and developed for the production of glycerol aerobically in this laboratory earlier, was used for the production of mead under microaerophilic conditions. This parental strain was further developed for high ethanol tolerance and production.

Cell growth. The culture was grown for cell mass at a pH of 5.4 and a temperature of 30° C in the following medium: 25 g/l glucose or honey mash, 5 g/l yeast extract (Gibco Laboratories, Madison, WI, USA) and 5 g/l malt extract (Difco Laboratories, Detroit, MI, USA). The cell growth was supported by aeration at a rate of 1 vvm and agitation at a rate of 200 rpm in glass fermentors with a capacity from 2 to 10 l.

The cells were harvested after log phase followed by washing with sterilized 9 g/l NaCl solution to remove medium ingredients. The cells were stored at −4° C until required for immobilization.

Immobilization. The cells were immobilized in calcium alginate gels (Qureshi and Tamhane 1985). A slurry of yeast cells (150 g/l, dry wt), sodium alginate (12.5 g/l) and charcoal (6 g/l) was extruded into 0.15 M $CaCl_2$ solution through a 20 gauge (measurement of thickness, British standard wire gauge) needle syringe to give beads of 3 mm diameter. Charcoal was added to provide for strength of the beads and to clarify the mead produced. These beads were matured overnight at 4° C (in this time the Ca^{2+} could replace the Na^+ completely in the beads). The beads were activated for one hour in 150 g/l honey mash at a pH of 5.4 and a temperature of 30° C before the fermentation of mead.

Fermentation Reactor. The activated beads were packed in reactors of various sizes (2.2×60, 4×40 and 8×80 cm), designed for alcoholic fermentations by calcium alginate immobilized yeast cells (Qureshi et al. 1987). The void volume fraction (ratio of void volume to total volume) in each reactor was 0.48—0.50 of the total volume. These reactors were fed at the bottom with a peristaltic pump thus getting product at the top of the reactor.

Fermentation conditions. Honey mashes (84% w/v reducing sugars) diluted to 180 g/l total reducing sugars with distilled water were sterilized and fed to the immobilized cell packed bed reactors at a pH of 4.5 and a temperature of 27—35° C. Samples were taken from the outgoing product under steady state and analysed for residual reducing sugars, ethyl alcohol and ethyl acetate. No nitrogen source was incorporated in the feed medium while producing mead. However nitrogen sources were required for reactivation of cells when their activity was reduced.

Analyses. The total reducing sugars, ethanol, ethyl acetate and dry cell mass were estimated by DNS method (Miller 1959), pyknometric method (Horwitz 1980), saponification method (Woodman 1941) and optical density method, respectively. Further ethanol and ethyl acetate were confirmed by gas chromatography equipped with Carbowax 20M (G.C. Lab. Biochemistry and Food Technol. Division, BARC, Bombay, India) glass column (6 feet × ¼ inch) and flame ionization detector under following conditions: column temperature 72° C, detector temperature 72° C, injection port temperature 90° C and carrier gas (N_2) flow rate 20 ml/min.

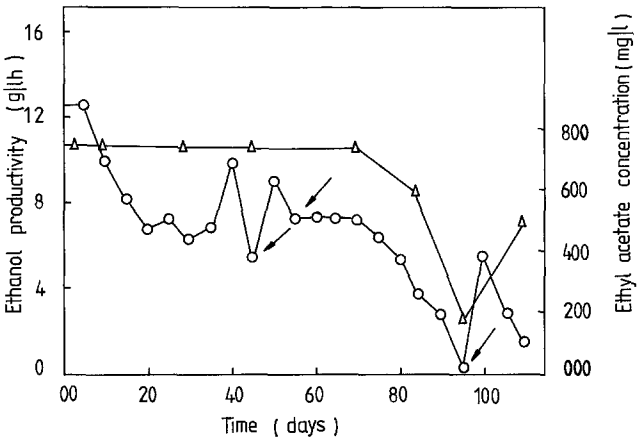

Fig. 2. Continuous mead production in immobilized cell packed bed column reactor of *H. anomala*. The dilution rate was 1 h^{-1}. (○) ethanol productivity; (△) ethyl acetate concentration. *Arrows* indicate the time of circulation of nutrient medium

Results

Experiments were carried out to find effect of feed rate on the conversion of sugars to ethanol and ethyl acetate. The packed bed reactor with immobilized cells produced a maximum ethanol concentration of 70 g/l and ethyl acetate of 730 mg/l at a dilution rate of 0.1 h^{-1} based upon void volume of reactor. At this dilution rate the effluent contained 43 g/l reducing sugars along with above products thus showing 76% (w/v) conversion of sugars to ethanol and ethyl acetate. The reactor showed a maximum alcohol productivity of 23.1 g/l·h at the dilution rate of 0.33 h^{-1} (Fig. 1). Batch growth of the free cells showed an ethanol productivity of 0.05 g/l·h with 280 mg/l ethyl acetate in the product.

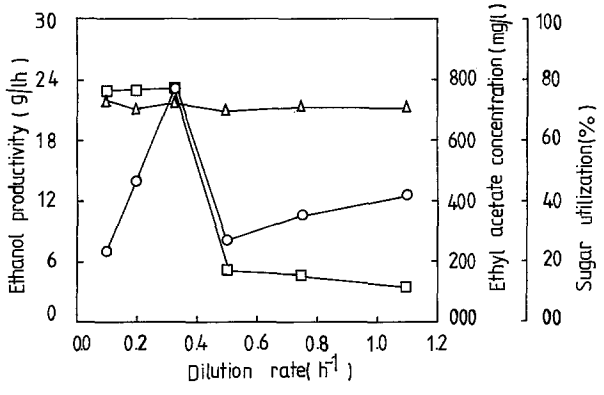

Fig. 1. Production of mead in immobilized cell column reactor of *H. anomala*. (○) ethanol productivity; (△) ethyl acetate concentration in product; and (□) sugar utilization

Since one of the advantages of immobilized cells is their long term operational stability, the once packed reactor was operated to produce mead continuously. The reactor worked for a total period of 110 days followed by disintegration of beads and their washout with the effluent. The cells required reactivation after a period of 44, 60, and 95 days. Therefore the nutrient medium as used for the growth of cells, was circulated for 24 h through the reactor followed by feeding the column with honey mash. The rates of ethanol production and concentrations of ethyl acetate

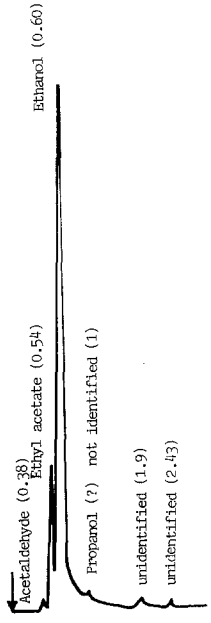

Fig. 3. Gas chromatogram of a mead produced by immobilized cells of *H. anomala*. Numbers in brackets indicate retention time in minutes

obtained in the product are shown in Fig. 2, while operating the reactor for 110 days. Figure 3 shows a chromatogram of a mead produced in this way. The results of the present system are compared with the earlier work carried out with immobilized cells of *S. cerevisiae* BRL-7 and *H. anomala* (Table 1). All the three reactors of dimensions 2.2×60, 4×40 and 8×80 cm gave similar results under identical conditions.

Discussion

The production of matured mead is a complicated process which requires aging to develop a large number of aroma compounds, many of them still unknown, after alcoholic fermentation. Some of these aroma compounds are dominating and contribute more to the flavour of a drink e.g. ethyl acetate. The development of these aroma compounds during aging process takes from a few weeks to a few years (Jorczyk and Wzorek 1977) and is of course an expensive process and sometimes results in spoilage due to contamination. In order to reduce the period of fermentation and the aging time, the immobilized and coimmobilized cells of *S. cerevisiae* BRL-7 and *H. anomala* were used in series reactors and single reactor, respectively (Qureshi and Tamhane 1986).

Since the growth of above two cultures for cell mass production for immobilization takes place in different media and under different environmental conditions, the process becomes difficult to operate. Also the immobilization of two cultures and operation of two series reactors is not an easy and industrially feasible process. In this view a single culture of *H. anomala* was used to produce mead containing ethanol and ethyl acetate in immobilized cell packed bed reactors. This system of mead production is compared with earlier reported systems of mead and ethanol production for alcohol productivity, maximum ethanol concentration achieved, and long term operational stability (Qureshi and Tamhane 1986; Qureshi et al. 1987).

The reactor packed once operated for a period of 110 days with intermittent regenerations of cell activity. This activity was possibly reduced either due to depletion of cofactors or inactivation of cells in absence of nutrients. The cells were reactivated by circulation of nutrient medium through the packed bed reactor. However, one of the possible reasons for cell reactivation may be cell proliferation inside the bead matrix. Williams and Munnecke (1981) also reported on similar phe-

Table 1. Production of mead by immobilized cells of *Saccharomyces cerevisiae* BRL-7, *Hansenula anomala* and coimmobilized cells of *S. cerevisiae* BRL-7 and *H. anomala*

Culture	Maximum alcohol and ethyl acetate concentration achieved			Maximum alcohol productivity achieved			Working life of reactor	References
	alcohol conc. (g/l)	ethyl acetate conc. (mg/l)	dil. rate (h^{-1})	alcohol productivity (g/l·h)	ethyl acetate conc. (mg/l)	dil. rate (h^{-1})	(days)	
Saccharomyces cerevisiae BRL-7 (For etOH)	90		0.33	51.1	—	1.67	130	Qureshi et al. 1987
Saccharomyces cerevisiae BRL-7	—	—	—	51.1	482	1.27	130 (unpublished)	Qureshi and Tamhane 1985, 1986
Saccharomyces cerevisiae BRL-7 & *Hansenula anomala* (series reactors)	54.5	—	0.33	61.3	616	1.36	—	Qureshi and Tamhane 1986
Coimmobilized *Saccharomyces cerevisiae* BRL-7 & *Hansenula anomala* (3:1 ratio)	110	—	0.67	80.2	400	2.15	—	Qureshi and Tamhane 1986
Coimmobilized *Saccharomyces cerevisiae* BRL-7 & *Hansenula anomala* (1:1 ratio)	101	—	0.67	63.0	500	2.00		Qureshi and Tamhane 1986
Hansenula anomala	70	730	0.10	23.1	680	0.33	110	This work

nomena of reactivation of cells. These authors also circulated nutrient medium for reactivation of cells through the packed reactors with immobilized cells of *S. cerevisiae* used for the production of ethanol. However, no reason has been suggested for the reduced cell activity. During long term operation the steady state production of ethanol and ethyl acetate may be maintained by regular feeding the nutrient medium when activity is reduced. In conclusion, this system of mead production is quicker than the traditional one, simple, easy to operate and works long. The problems of secondary fermentation and spoilage of mead during aging process are also eliminated.

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