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Production of Malt Vinegar by Immobilization Technique

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Barley malt, sSmi continuous, Acetous fermentation, Immobilization

A B S T R A C T

Barley malt wort was converted to ethanol by *Saccharomyces cerevisiae* strain 35 producing 5.2% (v/v) ethanol. This ethanol was used for vinegar production using encapsulated (Calcium alginate, agar, collagen and gelatin) cells of *Gluconobacter oxydans* NBRC 3432. All four encapsulation materials were statistically similar for acetic acid production and produced total acidity upto 4.66% (w/v). By semi continuous fermentation, the time of acetic acid fermentation was reduced to 14 days compared to 28 days of batch fermentation. In terms of economy of time, calcium alginate column was proved to be the best fermentation column among the four encapsulation materials with the fermentation efficiency of 85%.

Introduction

Natural vinegar is a quality food additive as it abounds in most of the essential amino acids contributed from its raw materials as well as fermenting micro-organisms. It is reported to possess ample medicinal values to cure aches and gastric troubles (Yamagishi K, *et al*., 1998). However, it generally ignored the consumer and the producer ends due to high cost and long fermentation time inherent with faulty production. In rural areas, the masses resort to traditional fermentation methods without using proper cultures and cultural conditions. Entrepreneurs hesitate to fund vinegar production ventures due to low efficiency of the process and the lengthy fermentation extending upto 2 months. Moreover, people are unaware of the good properties of natural vinegar and have inhibition including it in their daily diet. Those who use vinegar, fall prey to using synthetic vinegar due to its low cost but high risk of harmful toxic components in it. The natural vinegar, which is produced by fermenting a number of fruits and cereals, generally comes at high cost and hence falls out of the choice of consumers. Rice vinegar is popular in Japan and China while malt vinegar is favourite of the west. The scenario in India is dismal as the stores shelves are full of synthetic vinegar bottles well little or no availability of natural

vinegar in India especially the malt vinegar in North India where barley is available in abundance and its malt being used in beer industry. Hence barley malt may be taken as a choice substrate for production of natural vinegar.In order to make the manufacturing process suitable for a low cost cottage industry, it is essential to produce natural vinegar as a cost effective excercise by reducing its fermentation duration and probably the immobilization of microbial cells is the right answer.

Cell immobilization provides a means to improve upon the fermentation process by increasing biomass, option of reusability, protection of cells from toxic effects of low pH, temperature, inhibitors etc. It is a known fact that ionic/ hydrophobic interactions of the immobilization matrix induces increased stability of the cells and a buffered zone is provided by the immobilization materials (Brodelius P *et al.*,1986 and Durham D *et al*.,1994). Cell immobilization also helps in early clarification of the product. Further, the choice of immobilization material in the form of inexpensive easily available inert biological materials can help reduce the cost of the process. The present study was undertaken to develop an efficient process using free cells of *Saccharomyces cerevisiae* for alcoholic fermentation and immobilized cells of *Gluconobacter oxydans* for acetic acid fermentation. Different inert materials were encapsulated to test their suitability for immobilization of the *G.oxydans* cells for vinegar production from barley malt.

Materials and Methods

The yeast *Saccharomyces cerevisiae* strain 35, an isolate from brewery waste and the bacteria *Gluconobacter oxydans* NBRC 3432 isolated from rotten grapes were used for the dual alcoholic and acetic acid fermentation.

Barley malt was saccharified and wort preparing using the conditions standardized by response surface methodology. Allowing dextrans allowed to settle, the clarified wort was placed in 5 litre flasks each with 3 litre at 10.5°B and inoculated with 16–20 h grown culture of the yeast with viable cell population of 10^6 cfu ml^{-1}. Samples were drawn every 12 h and °B determined until the fermentation stopped as indicated by cessation of CO_2 evolution. Ethanol was determined by the method of Caputi A and Wright D (1969). The fermented wort, say malt wine was allowed to settle for 2 days at 4°C and the supernatant was siphoned off. The ethanol content of the wine was estimated to be 4.8 % (v/v). It was inoculated with the entrapped cells of *G.oxydans.* For entrapment, a cell paste (36 h grown cells of bacterial culture with cell count 3 x 10^6 cells /ml) was used. The count of yeast and bacteria (in beads) were calculated by serial dilution agar plate technique.

Ca –Alginate Beads

A 4% (w/v) sodium alginate solution were mixed and extruded through a syringe into 0.2 M $CaCl_2$ (anhydrous) solution to form the calcium alginate beads. The mixture was allowed to rest for 24 h and the beads were harvested by filteration

Agar Beads

A 2% solution of gum was prepared, sterilized and cooled to 45°C and bacterial paste was added to it. The molten preparation was dropped into ice-cold vegetable oil to produce an emulsion that was cooled to 5°C with gentle stirring. The mixture was allowed to rest for 24 h and the beads were harvested by filtration. The excess of oil was removed by five washings with distilled water.

Collagen Beads

Collagen is a natural animal protein and cannot form beads by itself. Therefore alginate beads were formed under mild conditions and its gelling property was made use of to prepare beads of collagen:alginate. Droplets of a mixture containing collagen (1.07–1.90 mg/ml) and alginate (15mg/ml) were discharged into a 15mg/ml solution of $CaCl_2$ (pH 7.2) to form spherical gel beads. The collagen: alginate beads so formed were aged in the same solution for 10 min. Subsequently, the gel beads were washed with PBS buffer (pH 7.2) containing 1.1% w/v $CaCl_2$. The beads were washed in the buffer containing 0.05% L-lysine for 10 to 15 sec. After repeated washing, with PBS, the beads were suspended in 50 mM sodium citrate (pH 7.2) for 10 min. The citrate-treated gel beads(where alginate has been liquefied by citrate) were washed with and resuspended in PBS buffer. The collagen was reconstituted by incubating the beads at 37°C for 2 h.

Gelatin Beads

These are intact native polymeric beads of calcium chloride and gelatin. A viscous solution was prepared by dissolving 50:50 mixture of sodium alginate and gelatin into a definite volume of distilled water with constant stirring. It was mixed with the bacterial cell paste and added dropwise by a syringe into 0.5M $CaCl_2$ solution, with gentle stirring. The beads so prepared were cured in the same solution for 48 h and were harvested by filteration.

Scanning Microscopy

Bacterial cell pellet (from one ml of the culture broth) was processed for scanning electron microscopy (SEM) for the record of shape, *size and arrangement of cells.* Conventional method was followed for the sample preparation Russel and Bozolla (1999).

Statistical Analysis

All the experiments of alcoholic and acetic acid fermentation were carried out in quadruplicate. The results of quadruplicate were filled in random block design of G Stat software (Cheema H *et al*., 2007). The CD 5% value of data fitted in Random Block Design were calculated to interpret the results.

Results and Discussion

The solution with desirability 1 was the basis of the fermentation. In this fermentation, 3.5l water was added to malt: water (1:4) ratio and saccharified as per the conditions designed by RSM. The saccharified malt wort (3l) was inoculated with 24 h old *Saccharomyces cerevisiae* strain 35 and incubated till the cessation of the fermentation in 7 days. Table1 represents the falling brix values and pH of the fermenting wort, which were recorded regularly at 1day interval.

The results show that the initial brix 10.5 ºB dropped to 1.8ºB after 7 days of fermentation. The maximum fall in brix upto difference of 7.2 ºB occurred during the first 5 days of fermentation whereas it was least during 6 and 7^{th} days of fermentation. Initial pH 5.5 dropped to 3.2 at the end of fermentation in 7 days. It is further reported that 3 litre wort was fully saccharified in 6 hours and 30 min and the recovered volume of the wort was 2.7 l representing 90% recovery. The total and the reducing sugars in the wort were 9.8 and 8.8 % respectively. The alcohol content of the malt wine at the end of fermentation was 5.2% (v/v) with the initial total acidity of 0.48 % (w/v (table 2).

Five sets of experiments were laid which included four sets of four types of bacteria laiden beads namely sodium alginate, agar, gelatin – alginate and collagen – alginate and one set of control run as submerged fermentation using free cells of bacteria as inoculums. All the experiments were performed using 400 ml malt wine supplemented with 5% mother vinegar, serving as fermentation medium for vinegar production. While the control run used 5% (v/v) inoculum of 36 h old free cell culture of *Gluconobacter oxydans*, the four sets of experiments with immobilized cells used 250 beads packed in a vertical column (glass burrette with 3cm diameter) through which malt wine was allowed to percolate at 5ml/min to constitute a flow cycle of 40 min. A temperature of 30 °C was maintained uniformly for optimum acetous fermentation. The acidity (both total and volatile) levels were estimated every alternate day and this practice continued by the course of fermentation until the total acidity reached 4.4% which was supposed to match 4.0% volatile acidity in general (Amerine M A *et al*.,1980).

The fermentation efficiency of acetic acid production was calculated as:

Volatile acidity %(w/v)/ {alcohol (%, v/v)x1.304}x100

Alcohol (%,v/v) = Actual ethanol (%,v/v) x 0.8

Vinegar produced was stored at 4°C, for 3–4 days, and the settled bacterial cells and sediment were separated. This partially clarified vinegar was bottled and pasteurized (using a water bath at 65°C for 30 min) and stored at room temperature.

The acidity levels were found increasing steadily as recorded after every set of 20 flow cycles at two days until it attained minimum 4.40 % total acidity. The sodium alginate beads column produced numerically the highest 4.44 % (w/v) which corresponds to 4.16% (w/v) volatile acidity at the end of 12 days of acetous fermentation (Fig.1 and table 3). Moreover, all the encapsulated materials were statistically similar in their acetic acid production potential. As shown in table 4, the cell count (cfu/g) was 9.2 $x10^8$ and the fermentation efficiency of the alginate column was reported to be 85%.When agar beads were used as immobilization support, they took 16 days to attain the acidity level of 4.42% and volatile acidity of 4.11% (w/v). The cell count of the agar beads were less than sodium- alginate beads i.e. 2.0 x 10^7 cfu/g and the fermentation efficiency of the agar beads column was 85.4 %. When gelatin –alginate and collagen- alginate beads were compared, it was observed that gelatin beads took 18 days to attain 4.42% (w/v) acidity while alginate – collagen beads took 24 days to complete the acetous fermentation. The fermentation efficiency of the gelatine-alginate column was 89% while it was 95% for collagen- alginate. Calcium alginate beads gave the best swelling ratio of 42.8 followed by agar (34), gelatin – alginate (18.4) and collagen- alginate (3.66) beads.

It was found an increase in alcoholic concentration, productivity as well as efficiency with an increase in pH from 4.0 to 5.0 and found that the optimum pH range for *Saccharomyces cerevisiae* was 4.5–5.0 (Yadav B S *et al*,. 1997). Later it was found that the alcoholic fermentation is affected by its inoculum size (10^6 – 10^7 cells / ml) (Kathuria K, 1999). It was reported that the optimum fermentation of malt brew with addition of 10 per cent inoculum of *S. cerevisiae* G (Kaur S *et al*., 2005). Variability of falling ºbrix and alcohol production under different treatments in our

study could possibly be the outcome of such behaviour of yeast. It was stated that fermentative metabolism of yeast is responsible for wine aroma, particularly aromatic quality linked to the presence of different quantities of ethyl ethers, fatty acids and higher alcohol esters (Houtman AC and Plesis CS, 1986). Several indigenous *Saccharomyces cerevisiae* strains produced higher quantities of ethyl esters and fatty acids in wine probably by better adaptation to the chemical and microbiological characterstics of the must (Leema *et al*., 1996).

Table.1 Change in °Brix and pH of Malt Wort during Alcoholic Fermentation

Day	°Brix	Fall in°Brix	pH	Fall in pH
1	10.5	-	5.50	-
2	8.5	2.0	5.00	0.5
3	6.7	1.8	4.80	0.2
4	4.2	2.5	4.20	0.6
5	3.3	0.9	3.80	0.4
6	2.4	0.9	3.30	0.5
7	1.8	0.6	3.20	0.1
8	1.8	-	3.20	-

Table.2 Saccharification and Alcoholic Fermentation Parameters of Malt

pH	5.5
Temperature of saccharification (^{0}C)	55
Substrate concentration (%)	17.5
Starch Concentration (%)	11
Total time for saccharification (h:min)	6.30
Recovery (l)	2.7
Total sugars (%)	9.8
Reducing sugars (%)	8.8
Cell count of yeast inoculum (cfu/ml)	$2x10^7$
Ethanol produced % (v/v) (8 days)	5.2
Residual total sugars (%)	1.2± 0.08
Residual reducing sugars (%)	0.8±0.071
Fermentation efficiency (%)	94.5

Fig.1 Surface Morphology of Calcium Alginate Bead

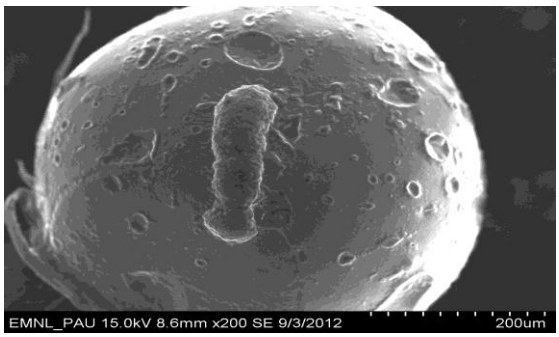


Table.3 Comparison of Immobilization Carriers for Progressive Acidity Production during Vinegar Fermentation

Carrier Days Of Acetous fermentation	Control		Collagen-alginate		Gelatin-alginate		Agar		Na- alginate		Corn cobs (treated)		
	Total acidity	Volatile acidity	Total acidity	Volatile acidity	Total acidity	Volatile acidity	Total acidity	Volatile acidity	Total acidity	Volatile acidity	Volatile acidity	Total acidity	Volatile acidity
0	0.32	0.10	0.60	0.11	0.62	0.42	0.61	0.41	0.51	0.32	0.11	0.62	0.21
2	1.00	0.72	0.92	0.50	1.32	1.11	1.00	0.80	1.21	0.94	0.92	1.21	0.72
4	1.21	1.00	1.00	0.60	1.93	1.65	1.52	1.34	2.52	2.12	2.11	1.92	1.25
6	1.73	1.43	1.31	1.00	2.14	1.84	2.12	1.94	3.11	2.93	3.42	2.51	1.92
8	1.92	1.72	1.72	1.34	2.34	2.11	2.83	2.52	3.52	3.21	3.90	2.82	2.23
10	2.21	1.94	2.01	1.76	2.91	2.64	3.21	2.94	4.12	3.83	4.00	3.10	2.72
12	2.51	2.10	2.50	2.10	3.51	3.11	3.72	3.54	4.44	4.16	4.21	3.48	3.15
14	2.83	2.53	3.00	2.78	3.95	3.62	4.12	3.94				3.72	3.37
16	3.01	2.82	3.42	3.00	4.22	3.92	4.42	4.11				4.10	3.80
18	3.33	3.00	3.73	3.42	4.42	4.10						4.47	4.10
20	3.82	3.41	4.00	3.75									
22	4.10	3.93	4.31	3.90									
24	4.32	4.12	4.42	4.10									
26	4.38	4.10											
28	4.52	4.23											

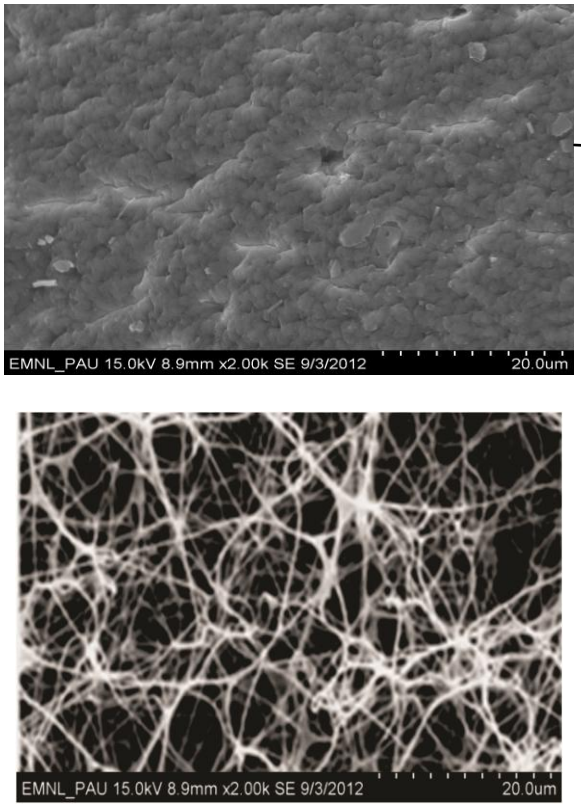
Volatile acidity of all the supports reported 0.251 CD (at 5%)

Table.4 Physical Characteristics and Performance of Immobilization Beads for Vinegar Fermentation

Bead type **Parameters**	**Na – alginate**	**Agar**	**Gelatin-alginate**	**Collagen-alginate**
Wet weight/ bead (mg)	6.92 [a]	7.00 [a]	9.72 [a]	4.21 [a]
Dry weight/bead (mg)	0.14 [b]	0.23[b]	0.52[b]	0.90[b]
Swelling ratio	48.2[c]	34.0[c]	18.4[c]	3.66[c]
No.of cells /bead	9.2 x10[8] [d]	2.0x10[7] [d]	5.2x10[6] [d]	4x10[6] [d]
Number of beads used	250	250	250	250
Initial ethanol of malt wine %(v/v)	5.21	5.21	5.21	5.21
Residual ethanol after fermentation %(v/v)	0.51	0.62	.75	1.00
Initial volume of malt wine(ml)	200	200	200	200
Final recovered volume of malt vinegar (ml)	130	120	100	100
Fermentation efficiency (%)	85	85.4	89	95

Swelling ratio = Wet weight/Dry weight -1
Where b (CD at 5%) = 0.288, a =.371, c= 1.31, d=.104

Fig.2 Physical Texture of Collagen Beads Before and afterTtreatment with Citrate Buffer



The count of the encapsulated bacterial cells at the end of fermentation was highest in the Na- alginate beads i.e. 9.2 $x10^8$ cfu /g and the least in the collagen -alginate beads i.e. $4x10^6$ cfu/g. It may be explained that the binding of the immobilized cells in collagen is probably due to hydrogen bonding and possibly the adsorption process involves the lysine residues (Cheetham, 1980). Formation of fibrous network in the collagen bead and amino acid lysine contritbe to the low cell count (Fig 2). The lysine, being negatively charged amino acid, repels the gram negative encapsulated *Gluconobacter oxydans* cells, which is responsible for low encapsulated bacterial cell count. The encapsulation count of the collagen alginate beads may be due to the macro porous nature of the collagen which may lead to the leakage of the microbial cells in the medium. The residual ethanol ranged from 0.58 to 1% (v/v) in different experiments. Similar observations have previously been made by Berraud C (2000) and Ory *et al*.(2004). On the other hand, fermentation efficiency with the alginate entrapped cells was low due to limited transport of substrate and oxygen across the beads and hence the diffusion gradient between gel matrix and the cells (D'Souza SF, 1989). However, a fermentation time of 28 days was still too long and thus the adsorbed cells were recycled. Earlier, a value of 20 times more vinegar productivity has been reported using a membrane bioreactor, rather than free cells (Mehaia MA and CheryanM, 1991). It was also reported that a vinegar productivity of 4.74 g/l /day using polyurethane absorbed cells of *A. aceti*. (Ory I *et al*., 2004).Calcium alginate beads gave the best swelling ratio due to the fact that alginate is a linear hydrophilic polymer and its increasing amount in the bead results in enlarged hydrohilicity of the network with the fixed ionic charges theory which resulted in larger swelling (Flory, 1953).

Conclusion

In the semi-continuous acetous fermentation trials using immobilized bacterial cells, collagen –alginate column gave the maximum fermentation efficiency of 95% but in terms of economy of process time, sodium alginate bead column proved to be the agar beads, alginate- gelatin beads and alginate -collagen beads. Sodium alginate bead column gave total acidity 4.66 % (w/v) with volatile acidity 4. 20 % (w/v) in the short span of 14 days with fermentation efficiency 85% as compared to the batch fermentation of 28 days.

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