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Vinegar Production from Barley Malt using Immobilized *Gluconobacter oxydans*

Naveet Kaushal[1*] and R. P. Phutela[2]

[1]Research Scholar, [2]Senoir Microbiologist,

Department of Microbiology, Punjab Agricultural University, Ludhiana

*Corresponding Author E-mail: leo.naveet@gmail.com

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ABSTRACT

Cell immobilization comprises the retention of metabolically active cells inside a polymeric matrix. In this study, the production of barley malt vinegar using immobilized Gluconobacter oxydans cells has proposed as a new method for commercial houses. 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 and agar) cells of Gluconobacter oxydans NBRC 3432. The optimized conditions for malt vinegar production were designed by response surface methodology. Both of 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 when compared to agar bead column with the fermentation efficiency of 85.5%.

Key words: *Barley malt, semi continuous, acetous fermentation, immobilization.*

INTRODUCTION

Vinegar is defined as "a liquid produced from raw material of agricultural origin, containing starch and sugars by the process of double fermentation, alcoholic and acetous and contains a specified amount of acetic acid". Vinegar, a traditional acidic condiment, is widely produced from rice, malt, apples, wine and various other agricultural material[5]. Vinegar production ranges from traditional methods employing wood casks and surface culture to submerged fermentation in acetator[13]. Acetic acid yield from fermented sugar is approximately 40%, with the remaining sugar metabolites either lost to volatilization or converted into other compounds[19]. 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[29]. It is reported to possess ample medicinal values to cure aches and gastric troubles [42]. Moreover, people are unaware of the good properties of natural vinegar and have inhibition including it in their daily diet.

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Those who use vinegar, fall prey to using synthetic vinegar due to its low cost but high risk of harmful toxic components in it. Starter cultures are commonly used in the food fermentation industry to predict and safeguard the product quality [30].These microbial starters have a significant role in the fermentation process [2].Yeast inoculation has been used extensively in the food industry to obtain a product with a predictable quality, including in the production of beverages such as wine [14,11,18]. In contrast, the use of acetic acid bacteria (AAB) as inoculum in the vinegar industry has traditionally been limited to the use of the mother of vinegar or back slopping. In such cases, the produced vinegar is a result of competition between microorganisms present in the base wine, particularly, wild AAB. The taxonomy of AAB has changed significantly in the last years. Currently, apart from *Acetobacter and Gluconobacter*[17] , other genera are classified as AAB. Different vinegars show different AAB profiles; nevertheless, species of the genera *Acetobacter*[38,16],*Gluconobacter*[34,37] and *Gluconacetobacter*[7,13]. *Acetobacter aceti* is a bacterium frequently used in the vinegar industry[36] because it immediately starts the fermentation process; however, when acetic acid concentrations exceed 5 %, other bacterial species take over the process[22]. On the other hand, *Gluconobacter* gives a distinctive flavour to vinegar and can oxidize ethanol to acetic acid under acidic conditions[34].Cell immobilization can be defined as the physical confinement of intact cells to a defined space to preserve the metabolic or catalytic activity. Immobilization mimics the enclosure or cell aggregation that normally occurs when microorganisms grow in natural environments, with the benefit of compartmentalizing the immobilized cell[4]. 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 using two inert materials for vinegar production from barley malt.

MATERIAL 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 was prepared 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 chemical oxidation method[6]. 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.

Bead Preparation

a) 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

b) 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.

Viable cells: The viable cells (cfu/ml) for the total number of bacterial cell inside the beads were counted at time zero (when the malt wine was inoculated) and every 24 h until the end of vinegar production . Ten beads (approx. 0.5 ml

of carrier beads) were placed on a glass filter to drain the solution. The beads were then transferred to a 5ml burette containing 3.5 ml of sterile distilled water. The liquid height was recorded to determine the increase in volume due to the beads. The beads were then crushed in sterilized water using a glass stick to recover the immobilized cells. The total number of *Gluconobacter oxydans* cells (cfu/ml) was determined by inoculating this cell suspension on GYC agar containing (in %): yeast extract 1, d-glucose 10, calcium carbonate 2 and agar 2 (Merck) for 24–48 h at 30 °C. **Chromatographic analyses**: Volatile compounds in the vinegar were analyzed directly, according to using a gas chromatography system (GC-17A; Shimadzu) with a flame ionization detector (GC–FID) fitted with DB Wax silica capillary column (30 m×0.25 μm×0.25 mm i.d.; J&W Scientific, Folsom, CA, USA) [39.]

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 [32.]

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[8.] The CD 5% value of data fitted in Random Block Design were calculated to interpret the results.

RESULTS AND DISCUSSION

The solution with desirability one suggested by response surface methodology, was the basis of the fermentation. To prepare the saccharification wort, 3.5l water was added to malt: water (1:4) ratio and it was inoculated with 24 h old *Saccharomyces cerevisiae* strain 35 . It is incubated till the cessation of the fermentation in 7 days. Fig. 1 represents the falling brix values and pH of the fermenting wort, which were recorded regularly at 1day interval. The wort is 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 1)

The bacteria laiden beads of sodium alginate and agar using 400 ml malt wine supplemented with 5% mother vinegar, served as acetous fermentation medium .Whereas control run used 5% (v/v) inoculum of 36 h old free cell culture of *Gluconobacter oxydans* in submerged fermentation . The 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[1].

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.

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 [40].. Later it was found that the alcoholic

fermentation is affected by its inoculum size (10^6 – 10^7 cells / ml)[19]. The optimum fermentation of malt brew with addition of 10 per cent inoculum of *S. cerevisiae* G[20]. Variability of falling ºbrix and alcohol production under these 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[17]. 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[22].

Sodium alginate beads column touched the total acidity of 4.12 %(w/v) in just ten days with fermentation efficiency of 85.5%whereas agar beads column yielded the same in sixteen days with fermentation efficiency of 85.0% (Fig 2). The cell count of alginate beads was 9.2 x 10^8 cfu/gm whereas in agar beads ut was 2.0 x 10^7 cfu/gm. The fermentation efficiency with the agar 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[10]. Earlier, a value of 20 times more vinegar productivity has been reported using a membrane bioreactor, rather than free cells[23]. It was also reported that a vinegar productivity of 4.74 g/l /day using polyurethane absorbed cells of *A. aceti* [27]. 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[15] (Table 2).

Physicochemical Characterization of malt vinegar

Although two bead columns were used for entrapment of bacterial cells but the column product with high fermentation efficiency (Calcium alginate bead column) was selected for physicochemical characterization Seventeen compounds were identified and quantified in the malt vinegar: aldehydes, higher alcohols, terpene , acetate, diether, furans, acids, ketone and ethyl esters[24,31,35] (Table 3). A total of 96 compounds were identified in sherry vinegar: 26 esters, 23 carbonyl com- pounds, 20 alcohols, 14 acids, 6 volatile phenols, 3 lactones, 2 ethers, 1 acetal and 1 terpene[5]. The flavour of vinegars is determined by a series of volatile constituents of three different origins: substrate, acetification and ageing. Although several major volatile compounds such as acetic acid, ethyl acetate and acetaldehyde contribute to the final aroma of vinegar, many other minor compounds with a wide range of polarities, solubilities and volatilities could help explain the complexity of the overall sensation, especially if the vinegar is produced from fruits[22,39]. Acetaldehyde (3.72 mg/L) was the only aldehyde found in the malt vinegar. It is a very volatile compound; its content tends to decrease during acetification because it is an intermediary metabolite during the conversion of ethanol to acetic acid and is thus converted to acetic acid by the same metabolic pathway [5]. At low levels, acetaldehyde gives a pleasant fruity aroma to wines; however, at high concentrations, it has a pungent, irritating odour [25]. Four higher alcohols were identified in the malt vinegar. Among them, 2-phenylethanol was present at high levels. Its presence may result in flowery and sweet notes [28], which could be considered as a positive feature in the malt vinegar. Acetoin (3-hydroxy-2-butanone) was also a unique ketone that was identified in the malt vinegar (149.64 mg/L). It is a characteristic product of acetification and its concentration is very high in traditional vinegars[29]. It was reported that the content of acetoin ranged from 18 mg/L in malt vinegar to 227 mg/L in apple wine[26].

Table1: 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

Table2: Physical characteristics and performance of immobilization beads for vinegar fermentation

Parameters \ Bead type	Na – alginate	Agar
Wet weight/ bead (mg)	6.92 [a]	7.00 [a]
Dry weight/bead (mg)	0.14 [b]	0.23[b]
Swelling ratio	48.2[c]	34.0[c]
No.of cells /bead	$9.2 x10^{8}$ [d]	$2.0x10^{7}$ [d]
Number of beads used	250	250
Initial ethanol of malt wine %(v/v)	5.21	5.21
Residual ethanol after fermentation %(v/v)	0.51	0.62
Initial volume of malt wine(ml)	200	200
Final recovered volume of malt vinegar (ml)	130	120
Fermentation efficiency (%)	85.5	85.0

Swelling ratio = Wet weight/Dry weight -1

Where b (CD at 5%) = 0.288, a =.371, c= 1.31, d= .104

Table 3: Concentration of volatile compounds identified in the malt vinegar by GC-FID

No. Compound	LRI	ϒ /(mg/l)	Odour quality
Aledehyde			
1. Acetaldehyde	709	3.72	Fresh,green
Higher alcohols			
2. 2-Heptanol	1329	11.48	Coconut-like,ketonis solvent-like,unpleasant
3. 2-Methyl-1-propanol	1079	7.35	Malty
4. 2-Methyl-1- butanol and 3-methly-1-butanol	1240	22.21	Malty,solvent/like
5. 2-Phenylethanol	1896	31.40	Flowery,honey/like
Terpenes			
6. α- Terpineol	1682	4.17	Pine/like,terpenoid-like
Acetate			
7. Phenylethyl acetate	1826	1.38	Apple-like,honey-like,rosy,sweet;flowery
Diether			
8. 1,1-diethoxyethane	755	1.63	n.d.
Furan			
9. Furfuryl alcohol	1639	14.81	n.d.
Acids			
10. Decanoic	2287	2.61	Waxy,tallow,ransid,soapy;fatty
11. Isobutyric	1546	6.38	Sweet,bitter;cheesy,ransid
12. Hexanoic	1850	1.20	Fatty acid-like,vegetable oil-;cheesy, sweet
13. Propionic	1523	2.77	Vinegar-like
14. Octanoic	2061	1.77	Fatty acid-like,vegetable oil-like;rransid,harsh
Ketone			
15. Acetoin	1309	149.64	Buttery,creamy,cheesy
Ethyl esters			
16. Ethyl acetate	816	179.38	Solvent-like,fruity
17. Ethyl octanoate	1398	148.23	Apple-like,fruity;sweet

LRI Linear Retension Index, n.d, not defined

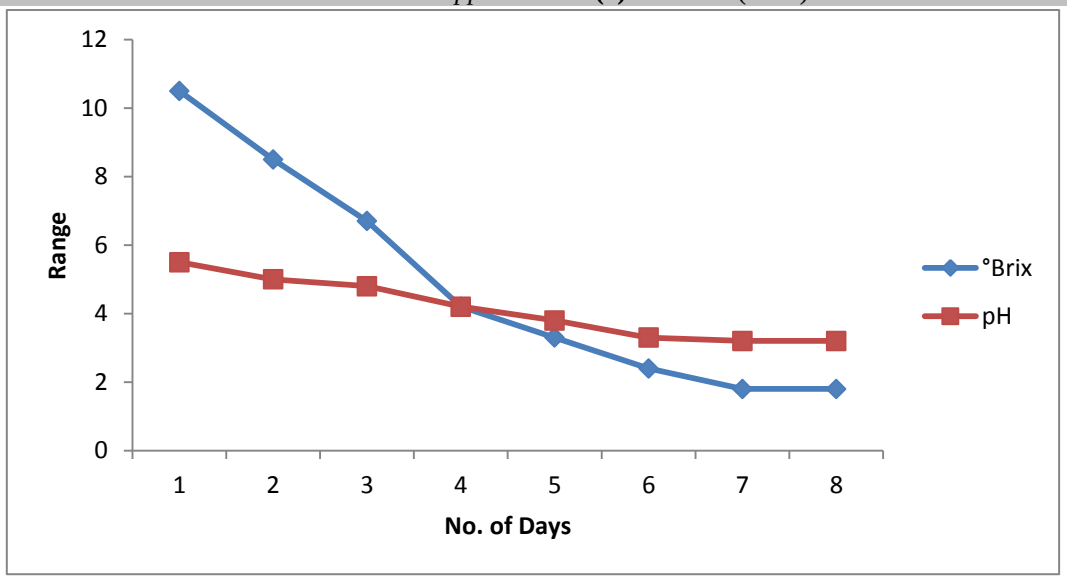

Fig. 1: Decrease in pH and Brix during alcoholic fermentation

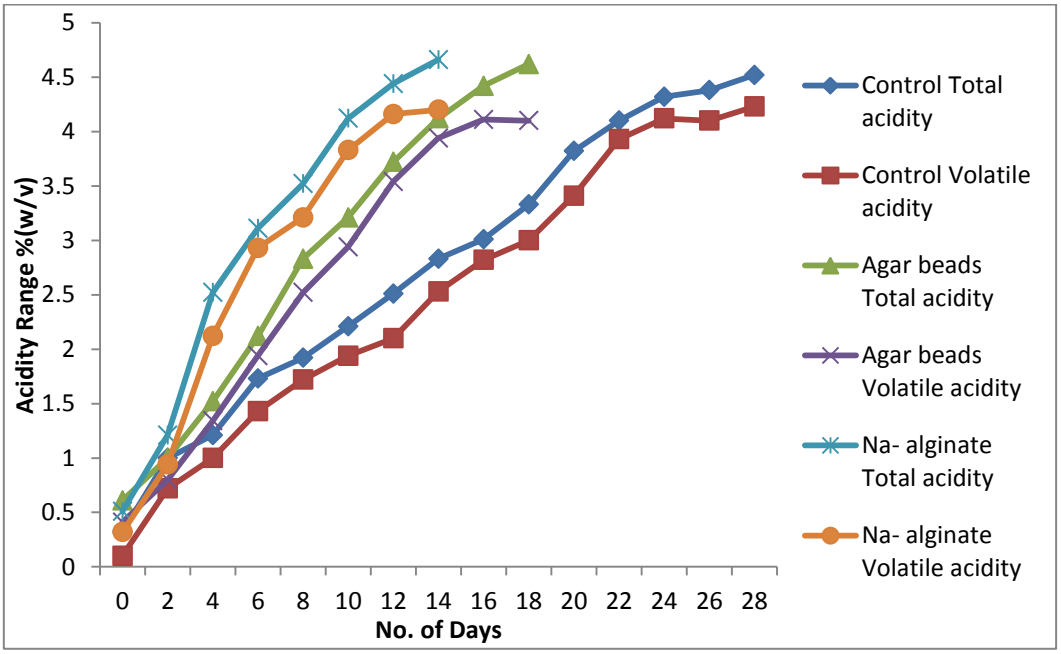

Fig 2: Shift pattern of acidity of immobilization supports during fermentation

CONCLUSION

In the semi-continuous acetous fermentation trials using immobilized bacterial cells, calcium –alginate column gave fermentation efficiency of 85.5% followed by 85% in agar beads. Although the two entrpement columns had not shown a significant difference in their efficiencies, but calcium alginate column was proved to be best in terms of economy of time. Furthur, the chemical analyses revealed that the malt vinegar had a high content of organic acids and volatile compounds, which add functional value and aroma to the vinegar. The technology proposed in this study is important and proved to be a viable technique for using harvest surpluses and obtaining products with higher market value.

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