Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

Value Addition to Cane Molasses, an Industrial Effluent via Sustainable Route

M.Kalyan Kumar [1], Udayasree[2], Subba Rao.D[3]

[1,2,3] Department of Chemical Engineering, JNTUA College of Engineering Ananthapuramu, Affiliated to JNTU Anantapur, Ananthapuramu.

(Received: 04 February 2024 Revised: 11 March 2024 Accepted: 08 April 2024)

KEYWORDS
cane molasses, gluconic acid, glucose oxidase, catalase, invertase.

ABSTRACT:
Cane molasses is an effluent released from sugar mills during the crystallisation of sugar, and the majority of this effluent is used in the production of ethanol. Gluconic acid (GA) is a valuable chemical whose salts find application in the pharmaceutical industry. Most of the gluconic acid present in India is produced by the electrolytic process, which is energy-intensive. The enzymatic route for producing gluconic acid from glucose present in cane molasses is low-energy consuming and sustainable as the enzymes in an immobilised state are recycled several times without much loss of activity. Invertase, glucose oxidase (GOD) and catalase (CAT) are used for producing gluconic acid. The optimal conditions for maximum yield of GA were determined by conducting several batch experiments using free and immobilised enzymes. The effective ratio for GOD and invertase was 1:2 for both free and immobilisation methods. GA production was maximum at 150 min with GOD of 36 units and 120 min using GOD 36 units, CAT 180 units together respectively. The right mix of alginate and enzyme solution was found to be 8:2 for the successful preparation of enzyme beads during the immobilisation process. 7 U/mL of invertase solution was effective for enhancing the glucose content in the molasses. The study confirmed the reusability of immobilised enzymes for GA production in spite of their reduced yield.

1. Introduction & Objectives

1.1 Cane Molasses:

Molasses is an effluent formed during crystallization of sugar from sugarcane juice. Molasses varies by sugar content in it, by method of extraction, and age of plant. It is a source of glucose which is a substrate for enzymatic production of Gluconic Acid (GA).

1.2 Gluconic acid:

Gluconic acid (GA) is an organic compound with IUPAC name pentahydroxy caproic acid ($C_6H_{12}O_7$) whose salts are important in pharmaceutical, food and other biological industries. It is a mild, neither caustic nor corrosive non-toxic and readily bio-degradable organic acid [1]. It is third largest in the global market of fermentation organic acid preceded by antibiotics and amino acids. Ability to bind with sodium, iron, and calcium gives a boost to gluconic acid market. Gluconic acid has many derivatives among which sodium gluconate used in textile dyeing and calcium gluconate used in pharma industries are potential drivers for the growth of the gluconic acid market.

1.3 Enzyme:

Enzymes are proteins within living cells that act as catalysts which increase rate of chemical reaction without being consumed. They can be used at the same time to carryout simultaneous reactions. Enzymatic methods do not generate environmental pollutants. Immobilization of enzymes is more beneficial when compared to that of cells as better control of the process is achieved with enzymes [2]. Glucose oxidase (GOD) enzyme is used for enzymatic production of gluconic acid from glucose. It catalyzes oxidation of glucose into GA and hydrogen peroxide.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOD} \text{gluconic acid} + H_2O_2$$

Invertase enzyme catalyzes the hydrolysis of sucrose into fructose and glucose.

$$C_{12}H_{22}O_{11} + H_2O \xrightarrow{\text{invertase}} C_6H_{12}O_6 + C_6H_{12}O_6$$

Catalase converts hydrogen peroxide into water and oxygen which further promotes the conversion of glucose to GA [3]. Catalase reaction produces oxygen which helps meet the oxygen requirement for GOD enzymatic reaction [4].

$$H_2O_2 \xrightarrow{\text{catalase}} \frac{1}{2} O_2 + H_2O$$

1.4 Objectives:

To produce gluconic acid (GA) by enzymatic method using the enzymes invertase, GOD and catalase in free and immobilised state.

To finalise process conditions for stable bead formation to immobilise the enzymes.

To identify optimum enzyme loadings for maximum conversion of glucose in the molasses.

To check the reusability of immobilised enzymes.

2. Materials and Methods

2.1 Substrate:

Cane molasses has been procured from a sugar mill located in Chittoor District of Andhra Pradesh.

2.2 Enzymes:

Enzymes glucose oxidase, invertase and catalase are procured from Sigma Aldrich in powder form.

2.3 Equipment:

Visible spectrophotometer, pH meter, auto clave, cyclo-mixer, heating mantle are made use of.

2.4 Pre-treatment of cane molasses:

Molasses is first clarified with distilled water [5]. Clarified molasses is pre-treated by different methods namely Potassium ferro cyanide treatment (KF), Sulphuric acid treatment (SA), Tricalcium phosphate treatment (TCP), Activated Carbon treatment (AC), Tricalcium phosphate hydrochloric acid treatment (TCPH) and Sulphuric acid+Activated carbon treatment (SA+AC) [6], for removal of organic and inorganic impurities.

2.5 Reagents:

All the chemicals used for assays, immobilization, pre-treatment are of analytical grade.

2.6 Assays:

Estimation of glucose is done by using DNS method [7]. The glucose concentration was determined from standard curve. Gluconic acid and glucono lactone were estimated using hydroxamate method [8]. GA+GL and GL concentrations were determined from standard plots. Gluconic acid in the sample was obtained by subtracting the gluconolactone (GL) concentration from the combined gluconic acid and gluconolactone (GA+GL) concentration.

2.7. Preparation of calcium alginate beads

The calcium alginate beads are prepared by drop wise addition of 10 mL alginate solution (4% w/v) into 20 mL of $CaCl_2$ solution (0.5 M) though a fine 21 gauge stainless steel needle. The distance of 6 cm between edge of needle and surface of calcium solution is maintained. The beads are left in the gelling medium for 15 min [9], then separated from solution through a stainless steel grid and left at room temperature for 15 min [10]. A mixture of 8 mL of alginate solution and 2 mL of enzyme solution is syringed to form beads containing the enzyme.

3. Results and Discussion

3.1 Reaction with free GOD enzyme:

Shake flask experiments are conducted using orbital shaker. Optimum conditions are maintained during the experiment (Table 3.1). The conversion of glucose has increased from 0 to 12.46% for SA+AC method, 9.98% for AC method, 8.08% for SA method, 21.91% for KF method, 18.22% for TCP method and 16.41% for TCPH method. KF treatment of cane molasses was observed to give the best results compared to other pre-treatment techniques this is because of effective removal of metal ions like Cu^{+2}, Fe^{+3}, Zn^{+2}, Mn^{+2}, Mg^{+2} and Ca^{+2} (Fig.3.1).

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

Table 3.1 Parameters for reaction with free GOD enzyme

S.No.	Parameter	
1	Temperature	55±5 °C
2	p^H	5.5±0.5
3	rpm	200±10
4	Enzyme solution	2.0 mL
5	Volume of sample	100 mL
6	Enzyme	GOD
7	Enzyme loading	72 U/100 mL

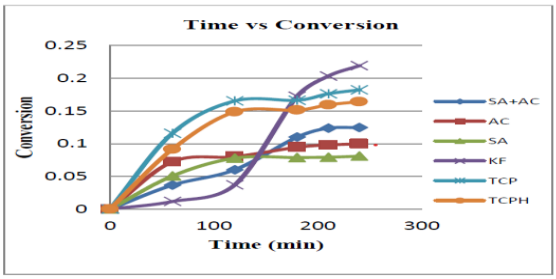

Figure 3.1 Conversion of glucose at different time intervals

3.2 Reaction of KF-treated molasses with free invertase enzyme:

Time-course experiments are conducted using orbital shaker with free invertase enzyme at around 40℃ [11]. Optimum conditions are maintained (Table 3.2) and the concentrations of glucose are measured.

Table 3.2 Parameters for reaction with invertase enzyme

S.No	Parameter	
1	Temperature	40 ± 5°C
2	pH	4.5±0.5
3	rpm	200±10
4	Initial concentration (C_{A0})	0.8767 mg/mL
5	Enzyme loading	Multiple loadings
6	Volume of sample	100 mL
7	Enzyme	Invertase

All enzyme loadings gave similar concentrations of glucose after 300 min. Among the different enzyme loadings it is better to use 7 U/100 ml of molasses because using higher loading of invertase has not shown any improvement (Fig.3.2).

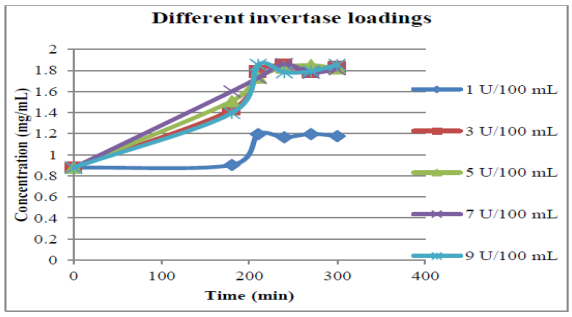

Figure 3.2 Concentration of glucose versus time for different invertase loading for KF-treated molasses

3.3 Reaction of KF- treated molasses with immobilized invertase:

Shake flask experiments are conducted using immobilized invertase enzyme at around 40℃ at optimum conditions (Table 3.3) and the concentrations of glucose are measured at different time intervals.

Table 3.3 Parameters for reaction with immobilized invertase enzyme

S.No	Parameter	
1	Temperature	40 ± 5°C
2	pH	4.5±0.5
3	rpm	200±10
4	invertase enzyme solution	2 mL
5	Volume of sample	100 mL
6	Enzyme loading	1,3,5,7,9 U/100 mL

The final glucose concentrations are obtained as 1.2645, 2.1022, 1.9066, 2.493 and 2.1531 mg/mL for immobilized invertase loadings 1, 3, 5, 7 and 9 U/100 mL respectively. Among all the different immobilized invertase enzyme loadings, better enrichment is observed at 7 U/100 mL (Fig.3.3).

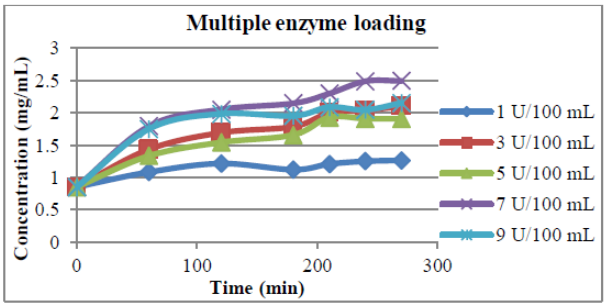

Fig 3.3 Glucose concentrations at different time intervals

3.4 Reaction of enriched KF-treated molasses with fresh GOD beads:

GOD enzyme (72 units/100 mL) in 1014 beads, each bead having 0.0710 units of GOD are prepared and added to 100 mL of enriched KF pre-treated molasses under optimum conditions (Table 3.4). Variation in the glucose concentration with time is measured and plotted (Fig.3.4). When fresh GOD beads are added to KF pre-treated molasses, the glucose concentration reduced from 1.2651 mg/mL to 1.2485 mg/mL (Fig.3.4).

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

Table 3.4 Parameters for reaction with GOD immobilized enzyme

S.No	Parameter	
1	Temperature	55±2^{0}C
2	pH	5.5±0.5
3	rpm	200±10
4	Enzyme solution	2 mL
5	Volume of sample	100 mL
6	Number of beads	1014 beads
7	Enzyme loading	72 units

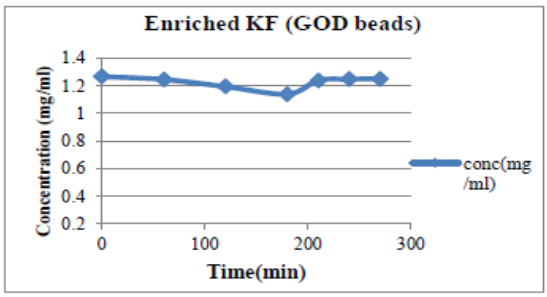

Fig 3.4 Concentration of glucose vs time plot for enriched KF pre-treated sample with fresh GOD beads

3.5 Reaction of enriched KF-treated molasses with reused GOD beads:

The GOD beads (72 units/100 mL) added to enriched KF pre-treated molasses were filtered and reused to observe the conversions. The glucose concentrations were measured at particular intervals of time and plotted. Reused GOD beads reduced glucose concentration from 1.275 mg/mL to 1.2562 mg/mL (Fig.3.5).

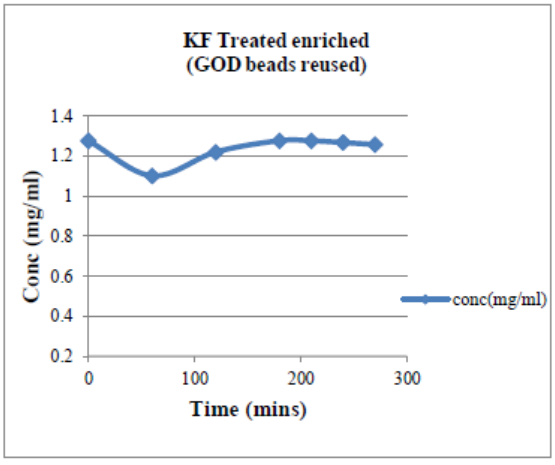

Fig 3.5 Concentration of glucose vs time plot for enriched KF pre-treated sample with reused GOD beads

3.6 Reaction of KF-treated molasses with GOD and invertase enzyme for finding optimum ratio:

Shake flask experiments are conducted using different GOD and invertase ratios. The ratios tested are 1:0.083, 1:1, 1:2, 2:1, 1:3, 3:1, 1:4, 4:1, 1:5, and 5:1 under optimum conditions (Table 3.5) [12].

Table 3.5 Parameters for reaction with GOD:Invertase of different enzyme loadings

S.No	Parameter	
1	Temperature	37±5^{0}C
2	pH	5.5±0.5
3	rpm	200±10
4	Enzyme loading (GOD:invertase)	Multiple loadings
5	Volume of sample	100 mL

The calculated conversions are obtained as 30.83%, 51.66%, 45.65%, 20.3%, 33.7%, 40.28% and 51.89% for 1:3, 1:4, 1:5, 1:0.083, 2:1, 1:1 and 1:2 respectively at 270 min. Among different GOD: invertase enzyme loadings 1:2 ratio was found to give best conversion (Fig. 3.6).

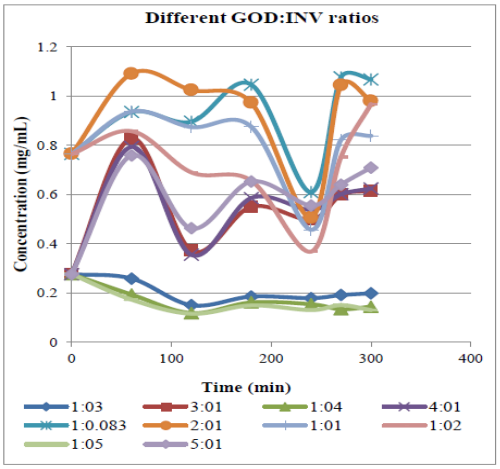

Fig 3.6 change in concentration of glucose with respect to time for different GOD:invertase loadings.

3.7 Reaction of KF-treated molasses with GOD and invertase enzymes beads:

Beads 1261 numbers having both GOD (72 units/100 mL) and invertase (144 units/100 mL) enzymes in the ratio 1:2 are prepared and added to 100 mL of KF pre-treated molasses under optimum conditions (Table 3.6). Variations in the glucose concentrations are measured.

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

Table 3.6 Parameters for reaction with GOD:invertase enzyme beads

S.No	Parameter	
1	Temperature	37±5℃
2	pH	4.5±0.5
3	rpm	200±10
4	Enzyme loading (GOD:Invertase)	1:2
5	Volume of sample	100 mL

3.8 Experimental results for reaction of KF-treated molasses with reused GOD and invertase enzymes beads:

The optimized GOD: Invertase ratio beads 1261 numbers are filtered and reused to observe the conversions. The glucose concentrations were measured at different time intervals using DNS method and plotted. For fresh GOD: invertase beads, the glucose concentration has reduced from 1.2485 mg/mL to 0.6909 mg/mL whereas for reused beads it has reduced from 0.9927 mg/mL to 0.7484 mg/mL. For optimized GOD: invertase beads, the glucose conversion was 44.66% and for reused one, it was 24.6% (Fig.3.7).

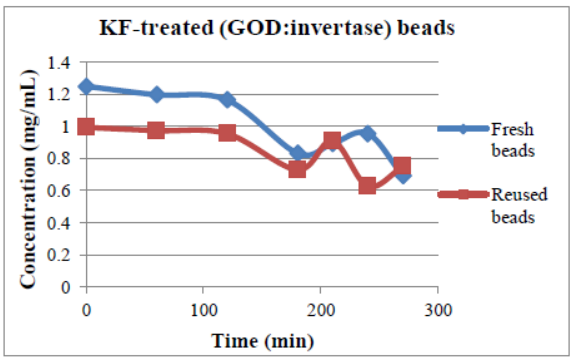

Fig 3.7 Concentration of glucose vs time plot for KF pre-treated sample with optimized GOD: invertase fresh and reused beads (1:2)

3.9 Reaction of KF-treated molasses with GOD and catalase:

Shake flask experiments are conducted using free GOD enzyme and catalase enzyme at 28℃. GOD and catalase are added in optimum ratio under optimum conditions (Table 3.7) [13]. The gluconic acid concentrations are measured and plotted.

Table 3.7 Parameters for reaction with GOD+ CAT enzyme

S.No	Parameter	
1	Temperature	26±2^{0}C
2	pH	5.5±0.5
3	rpm	200±10
4	GOD enzyme loading	36 units
	CAT enzyme loading	180 units
5	Activity ratio(GOD:CAT)	1:5
6	Volume of sample	100 mL

Maximum production of GA was obtained at 120 min by using both GOD and catalase, where it is 150 min in case of GOD enzyme (Fig.3.8).

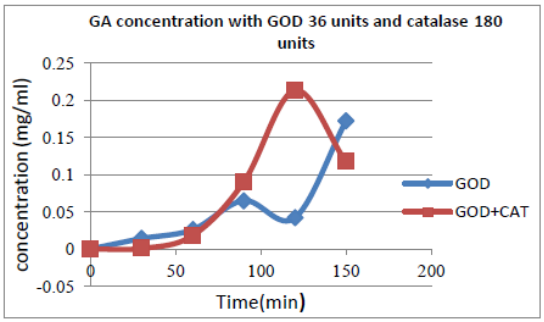

Fig 3.8 Concentration of GA with GOD and GOD+CAT

4. Conclusion:

GA was produced by enzymatic method by using invertase, GOD and catalase which showed maximum production at 120 min with free GOD and CAT ratio as 1:5. The optimum loading of invertase was 7 U/100 mL of sample and the optimum ratio of GOD and invertase was found to be 1:2. The bead formation was stable when 8 mL alginate solution was mixed with 2 mL enzyme solution, at needle height 6 cm and with mean diameter 0.1295 cm. Among different immobilized invertase enzyme loadings, 7 U/100 mL loading was observed to give best results. For optimized GOD: Invertase beads, the glucose conversion was 44.66% and for reused one, it was 24.6%. The study confirmed the reusability of immobilized enzymes for production of GA from glucose.

References:

1. Canete-Rodriguez, Ana M., et al. "Gluconic acid: properties, production methods and applications—an excellent opportunity for agro-industrial by-products

Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(3), 1694-1699 | ISSN:2251-6727

and waste bio-valorization." Process Biochemistry 51.12 (2016): 1891-1903.

2. Rao, D. Subba, and T. Panda. "Comparative analysis of different whole cell immobilized Aspergillus niger catalysts for gluconic acid fermentation using pretreated cane molasses." Bioprocess Engineering 11.5 (1994): 209-212.
3. Cui, Caixia, et al. "Genipin cross-linked glucose oxidase and catalase multi-enzyme for gluconic acid synthesis." Applied biochemistry and biotechnology 181.2 (2017): 526-535.
4. Jung, Dal-Young, Jules J. Magda, and In Suk Han. "Catalase effects on glucose sensitive hydrogels."Macromolecules 33.9 (2000): 3332-3336.
5. Rao, D. Subba, and T. Panda. "Critical analysis of the effect of metal ions on gluconic acid production by Aspergillus niger using a treated Indian cane molasses." Bioprocess Engineering 10.2 (1994): 99-107.
6. Kundu, Subir, et al. "Pretreatment of Indian cane molasses for increased production of citric acid." Biotechnology and bioengineering 26.9 (1984): 1114-1121.
7. Miller, Gail Lorenz. "Use of dinitrosalicylic acid reagent for determination of reducing sugar." Analytical chemistry 31.3 (1959): 426-428.
8. Lien,O.G. "Determination of gluconolactone, galactono lactone, and their free acids by hydroxamate method." Analytical Chemistry 31.8 (1959): 1363-1366.
9. Blandino, Ana, Manuel Macias, and Domingo Cantero. "Formation of calcium alginate gel capsules: influence of sodium alginate and CaCl2 concentration on gelation kinetics." Journal of bioscience and bioengineering 88.6 (1999): 686-689.
10. Blandino, A., M. Macıas, and D. Cantero. "Immobilization of glucose oxidase within calcium alginate gel capsules." Process Biochemistry 36.7 (2001): 601-606.
11. Tanriseven, Aziz, and Şenay Doğan. "Immobilization of invertase within calcium alginate gel capsules." Process Biochemistry 36.11 (2001): 1081-1083.
12. Silva, Aline Ramos da, Ester Junko Tomotani, and Michele Vitolo. "Invertase, glucose oxidase and catalase for converting sucrose to fructose and gluconic acid through batch and membrane continuous reactors." Brazilian Journal of Pharmaceutical Sciences 47.2 (2011): 399-407.
13. Godjevargova, T., Rajeshwar Dayal, and Ivaylo Marinov. "Simultaneous covalent immobilization of glucose oxidase and catalase onto chemically modified acrylonitrile copolymer membranes." Journal of applied polymer science 91.6 (2004): 4057-4063.