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Application of glucose oxidase for the production of metal gluconates by fermentation

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The present study deals with the application of glucose oxidase (GOX) for the production of metal gluconates by fermentation method. It provides a method for the conversion of glucose into gluconic acid and its derivatives using the enzyme glucose oxidase (GOX). Due to the presence of calcium carbonate in fermentation medium the gluconic acid is converted into calcium gluconate. Conditions like concentration of substrate, temperature, pH, fermentation period and different phosphate sources were optimized during fermentation. The maximum GOX activity was observed at 35°C (pH 5.5) after 44 h of incubation at 100 rpm. At the maximum enzyme activity, the percentage yield of gluconates are also maximum; both go side by side. Sulphuric and oxalic acids method were employed for the production of gluconic acid. Derivatives of gluconic acid that is, calcium lactate gluconate, sodium gluconate, potassium gluconate, zinc gluconate and copper gluconate were formed by using double displacement and direct methods. The direct method gave the better yield. The percentage yields were 73, 89.63, 81.93, 92.86 and 81.53%, respectively.

Key words: Glucose oxidase (GOX), metal gluconate, double displacement.

INTRODUCTION

Gluconates are salts of gluconic acid. Gluconic acid is an organic compound with molecular formula $C_6H_{12}O_7$. In aqueous solution at faintly acidic pH, gluconic acid forms the gluconate ion. Gluconic acid, gluconate salts and gluconate esters transpire widely in nature because such compounds come up from the oxidation of glucose (Henk, 2006). The glucono- delta-lactone is a food item with valuable features like even pH development and impartial taste forming a favoured additive for diverse foods like bread, mozzarella, sea food, meat, tofu etc. (Znad et al., 2003). Also, it is transformed to gluconic acid by fermentation method or through electrophoresis (Ramachandran and Fontanille, 2006). By *Aspergillus nigar* in an air lift reactor, high calcium gluconates production is attained as pellet form of cell growth at modest specific growth rate and biomass concentration (Anastassiadiss, 2007). The gluconates in electrolyte is improved by cooling the mother liquor obtained after recovery of product is incessantly reused in additional batches (Dowdells et al., 2010).

Gluconic acid consists of a six-carbon chain with five hydroxyl groups and at the end is a carboxylic acid group. In aqueous solution, gluconic acid originates in equilibrium with the cyclic ester glucono delta lactone. Including its derivatives, calcium lactate gluconate is a blend of calcium lactate and calcium gluconate. In pharmaceutical, it is extensively used as pharmaceutical calcium basis in food and beverages; the outstanding characteristics with high solubility and natural taste go ahead for new application in an extensive range of finest

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product (Jungbunzlauer, 2001). Also, it shows the highest solubility among the calcium salts frequently used for mineral enhancement, with a preliminary solubility of 20 to 30% (Chicage, 1999).

Sodium gluconate is the sodium salt of gluconic acid formed by gulucose fermentation. Its aqueous solution is subject to oxidation or reduction even at high temperatures. However, biologically, it is simply degraded and thus having no waste water difficulty. Potassium gluconate is a loosely bound salt of potassium and gluconic acid. It had been used in technical applications for example, as confiscating agent in textiles or for galvanic surface treatment (Life Science Research Office, 1978). Zinc salt of gluconic acid is called zincgluconate. It is useful as it has lower microbial profile and a more whole reaction, yielding a product with a longer shelf life (Caruso, 2007). The present study relates to the "microbial production of metal gluconates". More specifically, this invention provides a method for the conversion of glucose into gluconic acid and its derivatives using the enzyme glucose oxidase (GOX).

MATERIALS AND METHODS

The conversion of glucose into gluconic acid and its derivatives can be followed by: analysis of the glucose content, analysis of the gluconic acid content, confirmation of gluconic acid, conversion of gluconic acid into gluconate, determination of the percentage yield of metal gluconate.

Micro-organism

The strain of *Aspergillus niger* was grown on potato dextrose agar (PDA) and malt extract agar medium at pH 5.5.

Slants preparation

PDA was prepared by dissolving 40.0 g of PDA in 1000 ml of distilled water. To make the clear solution, medium was first boiled with constant stirring up to 15 to 20 min and then poured in the cotton plugged sterilized test tubes.

Sterilization

The medium in the cotton plugged test tubes and flasks were sterilized in the autoclave at 121°C and 15 lbs/inch^2 for 20 min and the test tubes were placed in slanting positions for 24 h after calving.

Inoculation

The slants were inoculated with the fresh strain of *A. niger* with the help of inoculums needle and incubated in the incubator at 37°C for 24 h. After every two weeks, propagation of strain on the fresh medium was continued. The pure and identified colonies of *A. niger* were stored in cold incubator/refrigerator at 4°C.

Fermentation media

Submerged fermentation was used for the production of GOX from *A. niger* in 250 ml shake flasks. The composition of fermentation medium was described in previous research project (Shazia et al., 2011).

Optimization of conditions

For submerged fermentation, the conditions of substrate (carbon source), concentration of substrate, pH of media, temperature and fermentation period were optimized.

Substrate (carbon source)

Different type of carbon sources/substrates were wormed for submerged fermentation like glucose, fructose, sucrose and dextrin.

Concentration of substrate (glucose, sucrose)

The carbon source (glucose, sucrose) which was obligated for fermentation as a carbon source was 4.0, 5.0, 6.0, 7.0, 8.0, 9, 10, 11, 12 and 13%.

Media pH

The pH of the media was accustomed with 1 M HCl and 1M NaOH. The adjusted pH was 4.0, 4.5. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, these flasks were brewed in shaking incubator at 30°C and 100 rpm for 45 h.

Incubation time/fermentation period

Growth media filled with 10% glucose in 250 ml of flasks were incubated in shaking incubator at 100 rpm and 30°C. They were taken out for analysis after 12, 22, 44, 66 and 88 h of incubation.

Temperature effect

Different conditions of temperature were 25, 35, 45, 65 and 75°C.

Effect of different PO_4 source

The different phosphate group were KH_2PO_4 and K_2HPO_4.

Extraction of enzyme

The extraction was same as described in our previous research project (Shazia et al., 2011).

Assay method

The enzyme assay followed the method of Shazia et al. (2011). Enzyme activity was calculated by the following formula:

$$\text{Activity of enzyme at 25°C} = \frac{\Delta A \times V}{I \; A_{290} \; \mu}$$

Where ΔA = the absorbance of the sample, V = volume of reaction mixture (4 ml), I = length of the cell (2.0 cm), A_{290}mM = molecular

Table 1. Effect of substrate on GOX.

Substrate	Absorption	Enzyme activity (µ moles HQ/min/ml)
Glucose	0.098	1.69
Fructose	0.067	1.160
Dextrin	0.0421	0.72
Sucrose	0.0194	0.336

absorption co– efficient of hydroquinone at 290 nm, (2.31), µ = glucose oxidase solution, (0.05 ml).

By fixing the values, the aforementioned equation will become:

Enzyme activity = 17.316 × ∆A µmole HQ / min / mg / ml (Ciucu and Patroescu, 1984).

Estimation of calcium gluconate

The filtrate contained gluconic acid which is converted into Ca-gluconate in the presence of $CaCO_3$ in aforementioned fermentation medium. Its analysis in supernatant liquid was made by the method of pharmacopoeia 1990.

Required chemicals

2.0 ml of 3M HCl, 20 ml of 0.05M EDTA, 15 ml of 1M NaOH, and 300 mg hydroxynaphthol blue (indicator) were used for the study.

Methods

1 ml of sample solution, 2.0 ml of 3M HCl were diluted with distilled water upto 150 ml with steady stirring. About 20 ml of 0.05 M EDTA was added from burette. 15 ml of 1 M NaOH and 300 mg of hydroxy naphthol blue indicator was added up to royal blue end point. Each ml of 0.05 M EDTA is corresponding to 2.004 mg of Ca-gluconate. The %age yield of Ca-gluconate was determined by the following formula:

$$\%\text{age yield} = \frac{\text{Ca-gluconate produced}}{\text{Glucose added}} \times 100$$

Preparation of gluconic acid from calcium gluconate

Oxalic acid method

5 g Ca-gluconate was dissolved in 25 ml boiled water.1.5 crystallized oxalic acid was dissolved firstly in minimum amount of water. Both the solutions were mixed at 50°C with constant stirring; the content was filtered to remove calcium oxalate. Gluconic acid was crystallized at 30°C in oven.

Sulphuric acid method

Sulphuric acid was used in place of oxalic acid to remove calcium as calcium sulphate and released the gluconic acid. 5 mg Ca-gluconate was dissolved in 10 ml distilled boiled water. This solution was placed in ice bath then 15.5 ml 30 N sulphuric acid was added dropwise, the content was stirred constantly for about 5 min, and filtered to remove Ca-sulphate. Gluconic acid solution was obtained as filtrate.

Conformational test for gluconic acid

5 ml warmed aqueous solution of gluconic acid and 1 ml freshly prepared distilled phenylhydrazine was taken. The mixture was taken in a test tube and the content was heated in water bath minutes. Crystals of gluconic acid phenylhydrazide were formed and melting point was noted.

Preparation of gluconic acid derivatives or gluconates

The derivatives of gluconic acid were prepared either from Ca-gluconate or directly from the gluconic acid using the methods: double decomposition and direct methods.

Double decomposition method: Metal gluconate was prepared by the double decomposition of metal sulphate and Ca-gluconate. 8.0 mg Ca-gluconate was added in 20 ml boiled water, stirred to dissolve it and treated with 8.0 mg metal sulphate with constant heating and stirring. Ca-sulphate was precipitated which was removed by filtration. Related metal gluconate was concentrated at constant low temperature. Ethanol was added to crystallize the metal gluconate, which was dried and weighted to calculate the yield.

Direct method: Related metal gluconate was prepared by the direct method from metal carbonate and gluconic acid. Metal carbonate (5.0 mg) was dissolved in 50% solution of gluconic acid (78 g corresponding to 156 ml). The solution was heated to remove the CO_2. Sodium gluconate was concentrated under vacuum at constant heating at 30°C. The contents were crystallized, dried and weighted to calculate the yield.

RESULTS AND DISCUSSION

The present studies show that the microbial production of gluconates such as sodium, potassium, zinc, copper and Ca-lactate gluconate were prepared in their best yield by using direct method approach. The percentage yields of metal gluconates were maximum at the maximum enzyme activity. Both results are shown in comparison with each other (Tables 1 to 3).

Calculations

Factor = each ml of 0.05 M EDTA equivalent to 2.004 mg of Ca-gluconate.

Table 2. Effect of glucose concentration on GOX.

Concentration of glucose (%)	Absorption	Enzyme activity (µ,moles HQ/min/ml)
4	0.052	0.901
6	0.063	1.098
8	0.098	1.69
10	0.051	0.894
12	0.024	0.42
14	0.081	0.22

Table 3. Effect of glucose concentration on gluconate production by *A. niger.*

Concentration of glucose (%)	EDTA used	Calcium gluconate produced
4	18.51	37.1
6	26.64	53.4
8	32.68	65.5
10	24.05	58.2
12	22.75	45.6
14	19.36	38.8

Table 4. Effect of fermentation period on GOX from *A. niger.*

Fermentation period (h)	Absorption	Enzyme activity (µ moles HQ/min/ml)
2	0.091	1.58
22	0.147	2.31
44	0.138	2.54
66	0.099	1.82
88	0.08	1.39

Calculation for maximum reading

1 ml of 0.05 M EDTA was equivalent to 2.004 mg of Ca-gluconate; 32.68 ml of 0.05 M EDTA = (2.004). (32.68) = 65.50 mg. Our results are in accordance with the results of Mischak (1985) and Petruccioli and Federici (1993); they reported that 8% glucose concentration enhanced the reaction, while Ray and Banik (1999) reported that 15% glucose concentration was affective (Tables 4 and 5).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 20.30 ml of 0.05 M EDTA = (2.004). (20.30) = 40.68 mg. Our results are antagonistic because in our results, 44 h fermentation period is effective for enhanced production of enzyme and gluconates; while Fiedurck (1998) reported 72 h fermentation period is effective (Tables 6 and 7).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 19.73 ml of 0.05M EDTA = (2.004). (19.73) = 39.53 mg. Our results are in accordance with Wiebel and Bright (1971) and Iedruck and Grumeda (2000) (Tables 8 and 9).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004

Table 5. Effect of fermentation period on Ca-gluconate production from *A. niger.*

Fermentation period (h)	EDTA used	Calcium gluconate produced
2	14.3	28.6
22	18.1	36.27
44	20.3	40.68
66	14.8	29.65
88	5.3	10.60

Table 6. Effect of pH on GOX production from *A. niger.*

pH	Absorption	Enzyme activity (µ moles HQ/min/ml)
4.0	0.054	0.936
4.5	0.080	1.391
5.0	0.098	1.432
5.5	0.087	1.692
6.0	0.058	1.01
6.5	0.025	0.44
7.0	0.011	0.199

Table 7. Effect of different pH for calcium gluconate production by *A. niger.*

pH	EDTA used (ml)	Calcium gluconate produced
4.0	16.1	32.26
4.5	18.8	37.71
5.0	17.2	34.48
5.5	19.7	39.53
6.0	15.7	31.52
6.5	14.0	28.07
7.0	13.3	26.65

Table 8. Effect of temperature on production of GOX by *A. niger.*

Temperature (°C)	Absorption	Enzyme activity (µ moles HQ/min/ml)
25	0.161	2.78
35	0.168	2.91
45	0.147	2.54
55	0.116	2.01
65	0.067	1.16

Table 9. Effect of temperature on Ca-gluconate production.

Temperature (°C)	EDTA used	Calcium gluconate produced
25	14.58	29.21
35	17.80	35.67
45	15.10	30.26
55	13.90	27.85
65	8.81	17.65

Table 10. Effect of different PO_4 sources.

Phosphate source	Glucose used (g/l)	EDTA used (ml)	Ca-gluconate produced	Percentage yield Ca-gluconate
KH_2PO_4	40	28.17	56.45	56.5
K_2HPO4	40	23.00	46.1	46.1

Table 11. Comparative percentage yield of metal gluconates.

Method	%yield Ca-lactate gluconate	% yield sodium gluconate	% yield pot-gluconate	% yield Zin-gluconate	% yield copper gluconate
Double decomposition method	68.04	87.35	78.97	81.01	80.00
Direct method	73.00	89.63	81.93	92.86	81.53

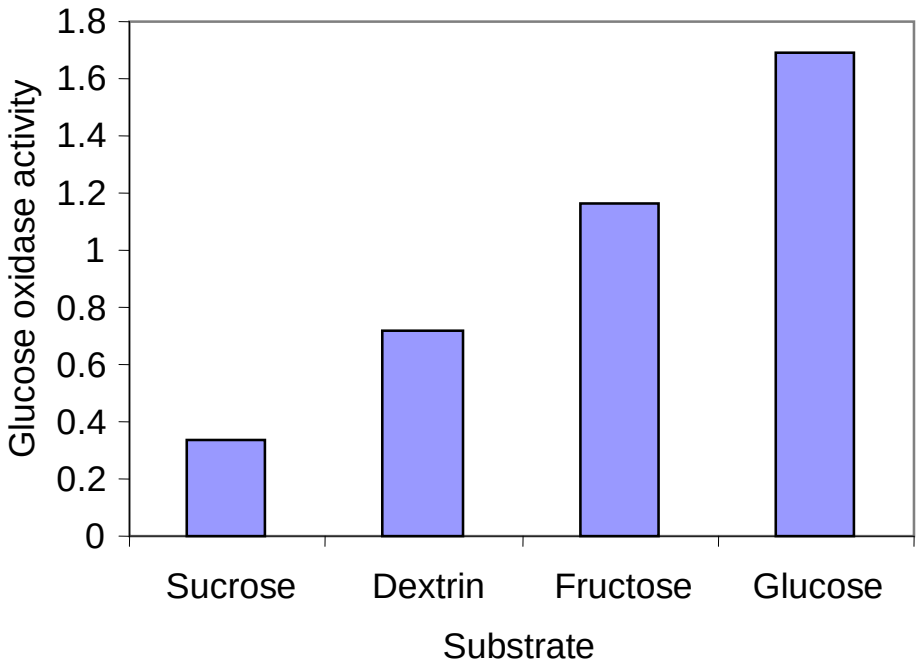

Figure 1. Effect of substrate on GOX.

mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 17.80 ml of 0.05M EDTA = (2.004). (17.80) = 35.67 mg (Table 10).

Calculation

Factor = each ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate.

Calculation for maximum reading

1 ml of 0.05M EDTA is equivalent to 2.004 mg of Ca-gluconate. 28.17 ml of 0.05M EDTA = (2.004). (28.17) = 56.45 mg. The results are in agreement with the work of Petruccioli and Federici (1993) (Table 11, Figures 1 to 10).

Conclusion

The present study shows that direct method gave better yield of metal gluconates as compared to double displacement method. These produced higher percentage

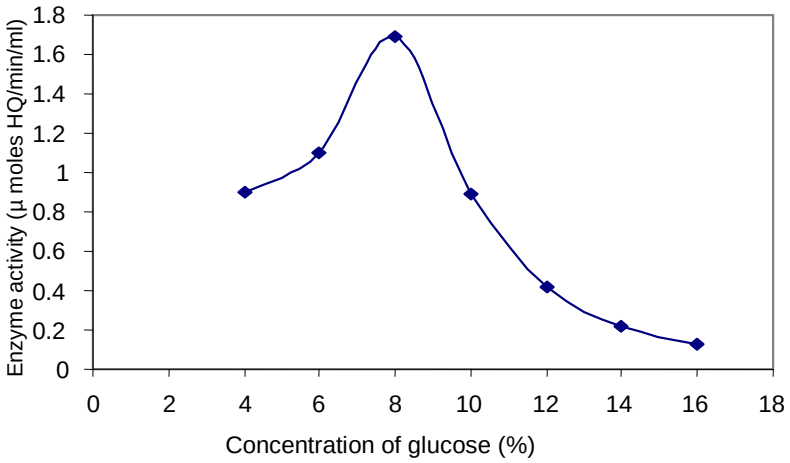

Figure 2. Effect of glucose concentration on GOX.

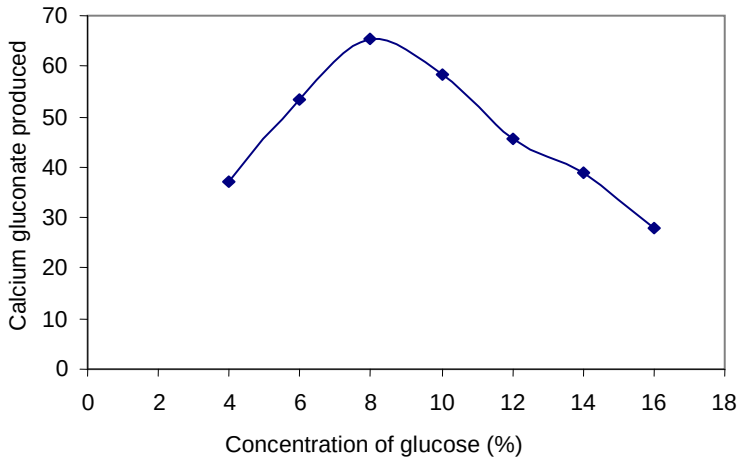

Figure 3. Effect of glucose concentration on gluconate production by *A. niger.*

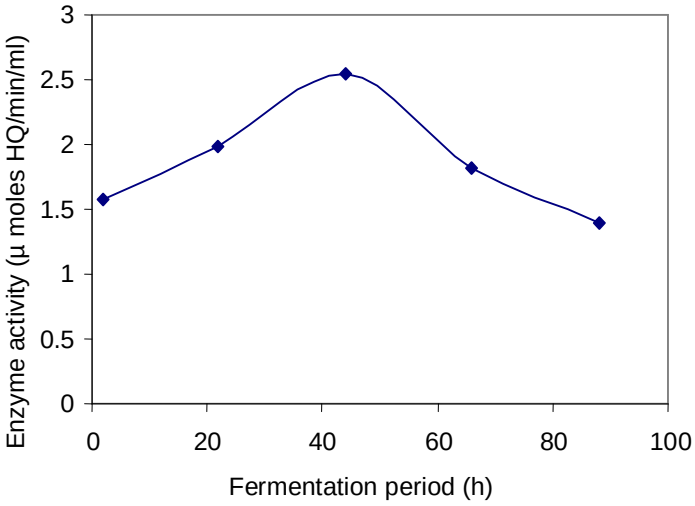

Figure 4. Effect of fermentation period on GOX from *A. niger.*

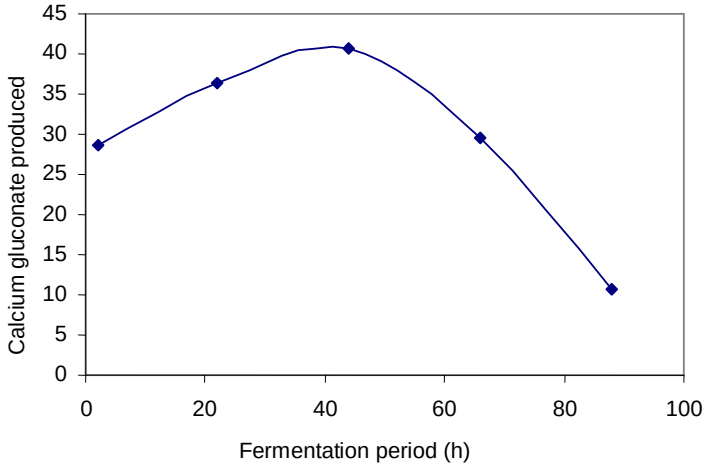

Figure 5. Effect of fermentation period on Ca-gluconate production from *A. niger.*

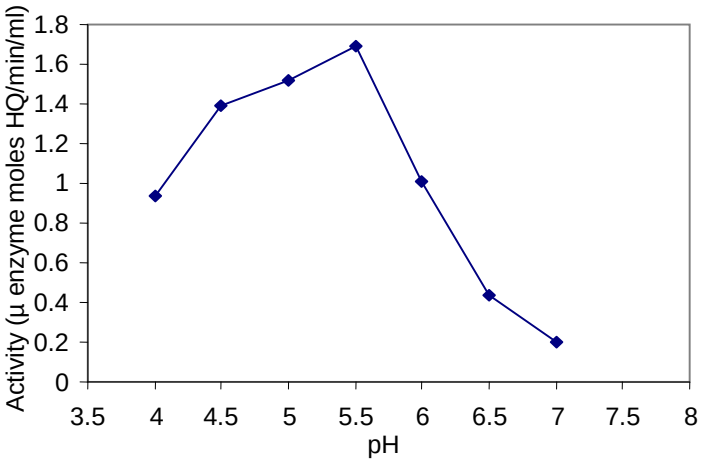

Figure 6. Effect of pH on GOX production from *A. niger.*

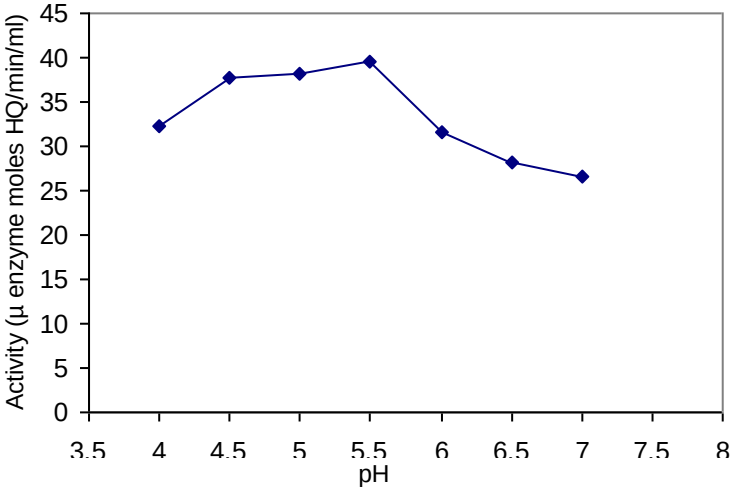

Figure 7. Effect of different pH for calcium gluconate production by *A. niger.*

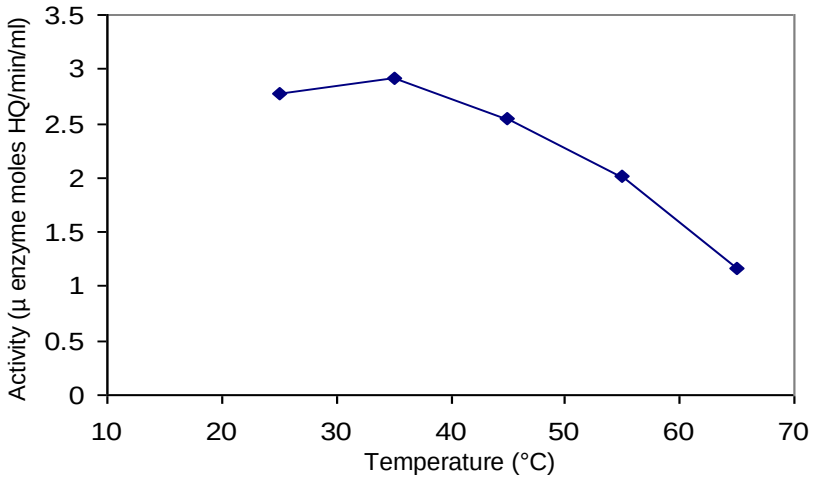

Figure 8. Effect of temperature on production of GOX by *A. niger.*

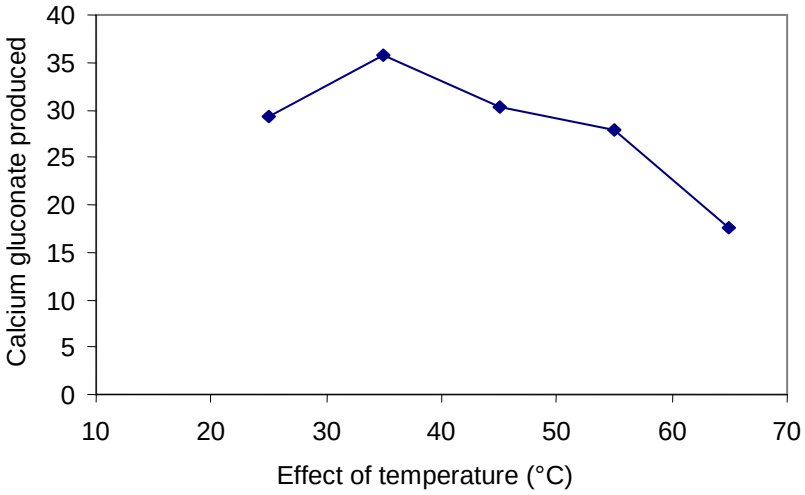

Figure 9. Effect of temperature on Ca-gluconate production.

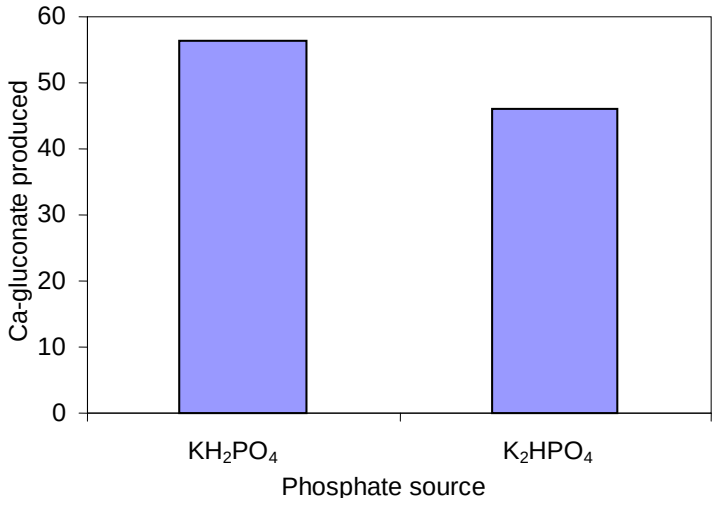

Figure 10. Effect of different PO_4 sources.

yields at the maximum enzyme activity. The fermentation method is a cost effective method.

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