

Studies on the production of glucose isomerase by *Bacillus licheniformis*

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This work reports the effects of some culture conditions on the production of glucose isomerase by *Bacillus licheniformis*. The bacterium was selected based on the release of 3.62 mg/mL fructose from the fermentation of glucose. Enzyme was produced using a variety of carbon substrates but the highest enzyme activity was detected in a medium containing 0.5% xylose and 1% glycerol (specific activity = 6.88 U/mg protein). Media containing only xylose or glucose gave lower enzyme productivities (specific activities = 4.60 and 2.35 U/mg protein respectively). The effects of nitrogen substrates on glucose isomerase production showed that yeast extract supported maximum enzyme activity (specific activity = 5.24 U/mg protein). Lowest enzyme activity was observed with sodium trioxonitrate (specific activity = 2.44 U/mg protein). In general, organic nitrogen substrates supported higher enzyme productivity than inorganic nitrogen substrates. Best enzyme activity was observed in the presence of Mg^{2+} (specific activity = 6.85 U/mg protein) while Hg^{2+} was inhibitory (specific activity = 1.02 U/mg protein). The optimum pH for best enzyme activity was 6.0 while optimum temperature for enzyme production was 50°C.

Keywords: glucose isomerase, carbon sources, nitrogen sources, metal ions.

INTRODUCTION

Glucose isomerase (GI) (E C 5.3.1.5) is one of the three tonnage value enzymes, amylase and protease being the other two¹. GI catalyzes the reversible isomerization of D- glucose and D- xylose to D-fructose and D- xylulose, respectively. Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant material and also aids in the bioconversion of hemicellulose to ethanol². Isomerization of glucose to fructose is of commercial importance in the production of high fructose corn syrup (HFCS). Silva *et al.*³, noted that the largest application of the technology of immobilized enzymes over the last 40 years has used GI.

GI is an intracellular enzyme produced from a range of genera principally *Streptomyces*, *Bacillus*, *Corynebacterium* and *Arthobacter* spp. and the organisms are grown on media containing glucose and/or xylose as the free sugar⁴. GI has also been produced from *Escherichia coli*^{5, 6} and from *Pichia pastoris*⁷. High fructose syrup produced by the activity of glucose isomerase is a useful sweetener; principally in carbonated beverages. According to Lawal *et al.*⁴, fructose has found a wide market in the United States in her soft drink industries.

Fructose is the sweetest of various naturally occurring sugars and there has been a large demand for it as an alternative to sucrose⁸. Glucose isomerase is used to produce high fructose corn starch. This process involves several separate enzymatic steps including liquefaction of corn starch by amylase, scarification by glycosidase and isomerization by glucose isomerase⁹. Fructose has long been recognized as a good alternative source of sugar due to its relatively high sweetness and other desirable physical and chemical properties. It has been reported to be 1.7 times sweeter than sucrose and it is widely used in food processing and formulation in pharmaceuticals¹⁰. Increasing demands for refined sugar, compiled with its rising price and the adverse effects of saccharine consumption on human health have necessitated the search for acceptable sucrose alternatives¹¹. Interests in converting glucose to fructose have attracted the atten-

tion of researchers who concerted their efforts towards maximizing fructose yield and minimizing the cost of all associated processes¹². This present work studies the effects of some culture conditions on the production of glucose isomerase by *Bacillus licheniformis*.

MATERIAL AND METHODS

Soil samples (ca. 20 g) was collected near a local corn mill into sterile polyethylene bags. A 10 g sample was dissolved in 90 mL of distilled water (pH = 6.5) contained in 500 ml Erlenmeyer flask and thoroughly shaken. Then, 1 mL of the sample was serially diluted in 9 mL 0.1% peptone water diluent. The diluted sample was plated onto Nutrient agar (Oxoid Ltd., UK) and the plates incubated at 35°C for 24 h. Pure bacterial cultures were obtained by streaking on fresh agar plates and were assigned arbitrary numbers. A loopfull of each culture was added into 100 mL medium containing glucose, 2%; yeast extract, 0.05%; KH_2PO_4 , 0.05%; K_2HPO_4 , 0.01% and $MgSO_4 \cdot 7H_2O$, 0.05% and incubated with shaking in a Gallenkamp orbital incubator for 24 h at 35°C. The medium was centrifuged at 2515 x g for 10 minutes in a Gallenkamp Junior centrifuge and the supernatant was assayed for fructose content. The isolate with the code number, A09 was selected for further work because it produced a maximum of 3.62 mg/mL fructose and was later identified as *Bacillus licheniformis* based on its morphological, physiological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology¹³.

Enzyme production and extraction

Bacterial cells were grown in 100 mL of medium described above and harvested by centrifugation at 2515 x g for 15 minutes and washed twice with 0.2 M phosphate buffer (pH 6.5). The washed cell suspension was disrupted by sonication for 10 min. using a Biologics Ultrasonic homogenizer Model 150VT (115V/60Hz). Following disruption, the mixture was centrifuged at 2515 x g for 15 minutes. The supernatant fluid was used to measure enzyme activity.

Enzyme assay

Enzyme solution (1 ml) in test tubes were incubated with 2 ml of phosphate buffer (pH 6.5) containing 1% glucose at $30 \pm 2^\circ\text{C}$ for 60 minutes followed by keeping the tubes in iced bath for 30 min. The amount of fructose formed was determined by the method of Kulka¹⁴. Briefly 0.5 mL of the reaction mixture in a test tube was added to 1.5 mL distilled water. Then 6 mL of ketose reagent [1:1 ratio of A (0.05 g resorcinol in 100 mL ethanol) and B [0.216 g $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 1000 ml HCl solution] was added. The content of the tube was mixed and immersed in a water bath (Kotterman, Bremen, Germany) at 80°C for 40 min. The tube was cooled in ice water and the absorbance measured in a Spectrumlab 23 A spectrophotometer at 480 nm. The absorbance was used to prepare a calibration curve with D- fructose (0–200 $\mu\text{g}/\text{mL}$). One unit of activity was defined as the amount of enzyme that released 1 μg of fructose per min. under the assay conditions.

Effect of carbon substrates on enzyme production

The basal medium for bacterial growth contained different carbon sources as indicated plus 0.5% yeast extract, 0.5% peptone in 0.2 M phosphate buffer (pH 6.5). The culture medium was inoculated with 2.5×10^6 colony forming units of *Bacillus licheniformis* and incubated for 24 h in a Gallenkamp orbital incubator at 35°C followed by enzyme extraction.

Effect of nitrogen substrates on enzyme production

Culture media contained different nitrogen sources as indicated plus 1% glucose in 0.2 M phosphate buffer (pH 6.5). The culture media were inoculated with 2.5×10^6 colony forming units of *Bacillus licheniformis* and incubated for 24 h in a Gallenkamp orbital incubator at 35°C followed by enzyme extraction.

Partial purification of the enzyme

The culture supernatant obtained after enzyme production and extraction was pooled and dialyzed overnight against 0.2M phosphate buffer (pH 6.5). Ammonium sulphate was added to the crude enzyme extract to 45% saturation, incubated for 8 hours with gentle mixing. The solution was centrifuged at $2515 \times g$ for 15 min. and the supernatant was subjected to further stepwise precipitation with ammonium sulphate to 65 and 75% saturation followed by centrifugation.

Effect of metal ions on enzyme production

The culture media contained different metal ions plus 1% glucose, 0.5% yeast extract, 0.5% peptone in 0.2 M phosphate buffer (pH 6.5). The culture media were inoculated with 2.5×10^6 colony forming units of *Bacillus licheniformis* and incubated for 24 h in a Gallenkamp orbital incubator at 35°C followed by enzyme extraction.

The influence of pH on enzyme activity

The effect of pH on activity of partially purified enzyme was determined by using buffer solutions of different pH (Phthalate-NaOH buffer pH 4.0–5.5, and Tris-Maleate buffer 6.0 to 8.5) for enzyme assay. The buffers were used at a concentration of 0.1 Mol/L. The pH activity profile of the enzyme was determined by incubating 0.5

mL of the enzyme contained in test tubes with 0.5 mL of 1% (w/v) glucose prepared in buffers of different pH values (4.0–8.5) at 40°C for 2 h. The reaction was stopped by placing the tubes in iced water and the enzyme activities were determined.

The influence of temperature on enzyme activity

The influence of temperature on partially purified enzyme activity was studied by incubating 0.5 mL of the enzyme solution contained in test tube and 0.5 mL of 1% glucose solution prepared in 0.2 M phosphate buffer (pH 6.5) for 3 h at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C) in a thermostatic water bath (Kottermann, Bremen, Germany). The reaction was stopped by placing the tubes in iced water and the enzyme activities were determined.

Assay procedures

Protein content was estimated by the method of Lowry et al.¹⁵, using bovine serum albumin (Sigma-Aldrich) as a standard. Fructose concentration was determined by the method of Kulka¹¹ using 50–200 μg fructose as standard.

RESULTS AND DISCUSSION

In a screening programme for the selection of the best glucose isomerase producer, twenty isolates were tested and released between 0.11 and 3.62 mg/mL fructose. A strain of bacteria designated A09 was chosen as a potent GI producer as determined quantitatively based on the release of 3.62 mg/mL of fructose from glucose contained in the assay medium (Table 1). From the morphological, biochemical and physiological characteristics, the isolate was identified as *Bacillus licheniformis*.

Although all the carbon substrates supported enzyme production by the bacterium, the best carbon substrate was a combination of 0.5% xylose and 1% glycerol (enzyme activity = 6.88 U/mg protein). Medium containing 0.5% xylose caused the production of only 4.6 U/mg protein while bacterial cultivation in the medium containing 0.5% glucose produced only 2.35 U/mg protein. In general, media containing xylose either alone or with glycerol caused higher enzyme activity than media containing only glucose or with glycerol (Table 2). GI

Table 1. Quantitative release of fructose by the isolates

Isolate	Fructose [mg/mL]
AO1	0.42
AO2	0.13
AO3	0.23
AO4	1.63
AO5	2.22
AO6	0.18
AO7	0.93
AO8	1.62
AO9	3.62
AO10	0.11
AO11	2.16
AO12	1.14
AO13	2.10
AO14	2.30
AO15	0.16
AO16	3.14
AO17	0.56
AO18	2.11
AO19	3.08
AO20	1.55

production from this work was compared to the data on GI production reported by other authors. Deshmukh et al.¹⁶, obtained the highest yield of 0.40 U/mg protein when xylose and sorbitol were used as carbon sources for enzyme production at a final pH of 8.0. Chou et al.¹⁷, reported the best yield of 1.45 U/mg protein when the mycelia of *Streptomyces* species grown in 1% xylose were subjected to heat treatment in the presence of 0.5 mM Co²⁺.

GI is an inducible enzyme and xylose is the most potent inducer of the enzyme¹⁸. The presence of D-xylose significantly increased the enzyme productivity by *Streptomyces* spp.¹⁹. Replacement of D-xylose with glucose reduced GI production. Kwakman and Postma²⁰ reported that the presence of glucose in microbial growth media caused reduction in the specific activities of the enzymes involved in the catabolism of other carbon sources. This could be attributed to the repressor effect of glucose on GI synthesis.

Table 3 shows the effect of nitrogen sources on enzyme activity by the bacterium. The best promoter of enzyme activity by the bacterium was generally organic nitrogen sources when compared to inorganic nitrogen sources. Inorganic nitrogen sources caused lower enzyme activity. Yeast extract caused the production of best enzyme yield (5.24 U/mg protein) by the bacterium. Replacement of

yeast extract with other nitrogen sources gave lower enzyme yields and the lowest yield was observed in the medium containing NaNO₃ as the sole nitrogen source (2.44 U/mg protein). The effects of nitrogen substrates on GI production by microorganisms have been reported²¹. Peptone, tryptone and yeast extract supported maximum growth and enzyme production by *Streptomyces thermotrophicans* and organic nitrogen sources were observed to support the highest GI production when compared to inorganic nitrogen sources¹⁶. Yassien and Jiman-Fatani²¹ reported that a combination of corn steep liquor (2%) and yeast extract (1%) resulted in an increase in GI production by about 3 to 4 times higher than other nitrogen sources tested.

As shown in Table 4, the tested ions promoted enzyme activity in varying degrees. The best enzyme activity was observed in medium containing Mg²⁺ followed by Mn²⁺ which gave enzyme specific activities of 6.85 and 5.20 U/mg protein respectively. The lowest enzyme productivity was observed in the medium containing Hg²⁺ with enzyme productivity at 1.02 U/mg protein. Glucose isomerase typically requires the presence of divalent metal cations such as Mg²⁺ or Co²⁺ as essential cofactors for their catalytic activity²². Kasumi et al.²³, found that Co²⁺ gave only 56% reaction activity as compared to Mg²⁺, but Ryu et al.²⁴, reported that the addition of Co²⁺

Table 2. Effects of carbon substrates on glucose isomerase activity of *Bacillus licheniformis*

C-source	Specific activity [U/mg protein]
Xylose (0.5%)	4.60 ±0.848
Glucose (0.5%)	2.35 ±0.778
0.5% xylose + 1% glucose	3.51 ±1.824
1% xylose + 0.5% glucose	2.12 ±0.735
0.5% xylose + 0.5% glucose	3.80 ±0.424
0.5% xylose + 1% glycerol	6.88 ±1.358
1% xylose + 0.5% glycerol	5.22 ±0.877
0.5% xylose + 0.5% glycerol	5.46 ±0.622
0.5% glucose + 1% glycerol	3.13 ±1.485
1% glucose + 0.5% glycerol	2.61 ±0.566
0.5% glucose + 0.5% glycerol	2.80 ±0.410

Mean ± SD of duplicate experiments

Table 3. Effects of nitrogen substrates on glucose isomerase activity of *Bacillus licheniformis*

N-source	Specific activity [U/mg protein]
Peptone	4.86 ±0.184
Yeast extract	5.24 ±0.311
Tryptone	3.62 ±0.806
Malt extract	3.21 ±0.325
Corn steep liquor	3.64 ±0.792
Urea	2.81 ±0.509
NaNO ₃	2.44 ±0.537
KNO ₃	2.64 ±0.778
(NH ₄) ₂ Cl	3.29 ±0.169
(NH ₄) ₂ SO ₄	3.56 ±0.071

Mean ± SD of duplicate experiments

Table 4. Effects of metal ions on glucose isomerase activity of *Bacillus licheniformis*

Metal ion [0.5 mM]	Specific activity [U/mg protein]
Control (No metal ion)	2.04 ±0.099
BaCl ₂	3.11 ±0.024
CaCl ₂	2.56 ±0.707
MnCl ₂	5.20 ±0.552
ZnCl ₂	2.93 ±0.325
CuCl ₂	3.64 ±0.028
CoCl ₂	4.68 ±0.466
HgCl ₂	1.02 ±0.057
MgSO ₄ · 7H ₂ O	6.85 ±0.382
AgNO ₃	2.39 ±0.877

Mean ± SD of duplicate experiments

into a culture medium stimulated the formation of GI. Treatment of purified enzyme with EDTA resulted in an almost complete loss of enzyme activity but the activity was restored by the addition of metal ions²⁵.

The effect of different buffers and pH on enzyme activity is shown in Figure 1. The enzyme was active over the whole range from pH 4.0–8.5 with maximal activity at pH 6.0 at which the enzyme retained 100% of its activity. Lowest activity was observed at pH 8.5 at which the enzyme lost 62% of its activity. Ryu et al.²⁴, showed pH 8.3 to be optimal for glucose isomerase production while Danno²⁶, Chen et al.¹⁸, and Yassien and Jiman-Fatani²¹ reported pH optima at 7.0 with the following microorganisms: *Bacillus coagulans*, *Streptomyces flavogriseus* and *Streptomyces albaduncus* respectively. Lawal et al.⁴ reported pH optimum at 10.0 with *Bacillus* spp. The optimum pH for GI production was in the range between pH 7.0–9.0⁹. The optimum pH for GI production was slightly acidic, pH 6.9 for *Streptomyces* species⁸.

The effect of temperature on enzyme activity showed 50°C as optimum temperature. The enzyme showed 100% activity at 50°C and lowest activity at 25°C at which 58% of its activity was lost. This is in line with the report of Gaily et al.¹², that during enzymatic isomerization of glucose, temperature is preferably maintained within the range of 20–90°C and the highest activity was obtained within

50–75°C. Optimal enzyme activity of glucose isomerase was observed at 42–43°C by Ryu et al.²⁴, while Yassien and Jiman-Fatani²¹ reported a suitable temperature for microbial growth and GI production in the range of 25–35°C for *Streptomyces albaduncus*.

CONCLUSION

The stimulatory effects of glycerol on glucose isomerase activity by *Bacillus licheniformis* was observed in media containing different carbon sources. Yeast extract was found to be the most suitable nitrogen source for enzyme production. Among the metal ions tested for enzyme activity, magnesium sulphate gave the best enzyme activity of 6.85 U/mg protein while the lowest enzyme activity of 1.02 U/mg protein was produced with mercury chloride. The enzyme activity produced at pH 6.0 was higher than at other pH levels. The optimum temperature for enzyme activity was found to be at 50°C. It would be possible to produce commercial amounts of this enzyme using the test bacterium, however, further kinetic studies on enzyme production need to be studied.

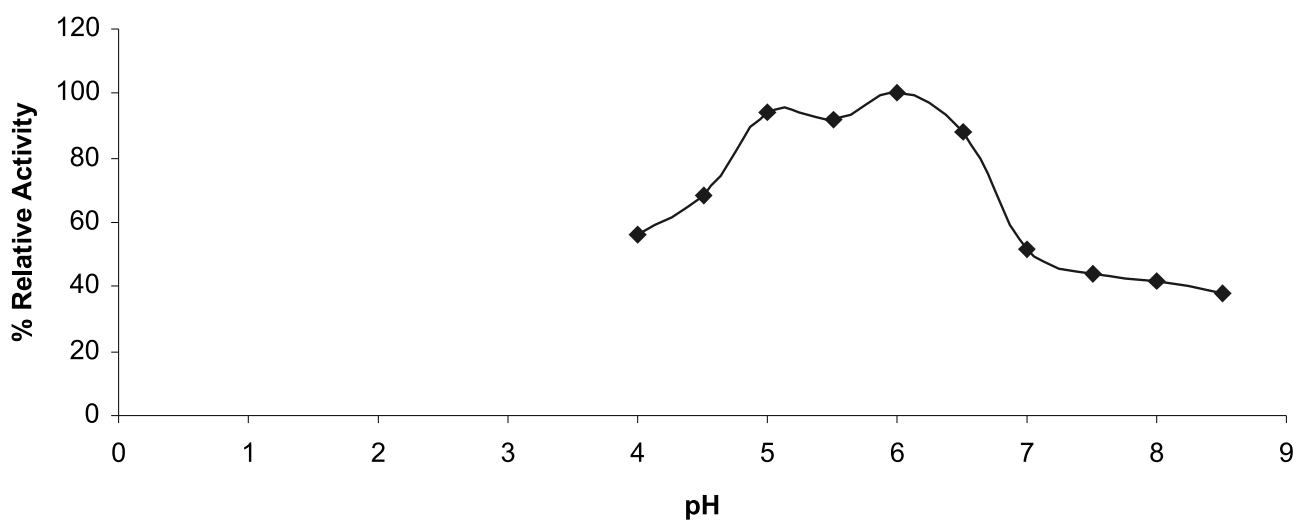


Figure 1. Effects of initial pH on glucose isomerase activity of *Bacillus licheniformis*

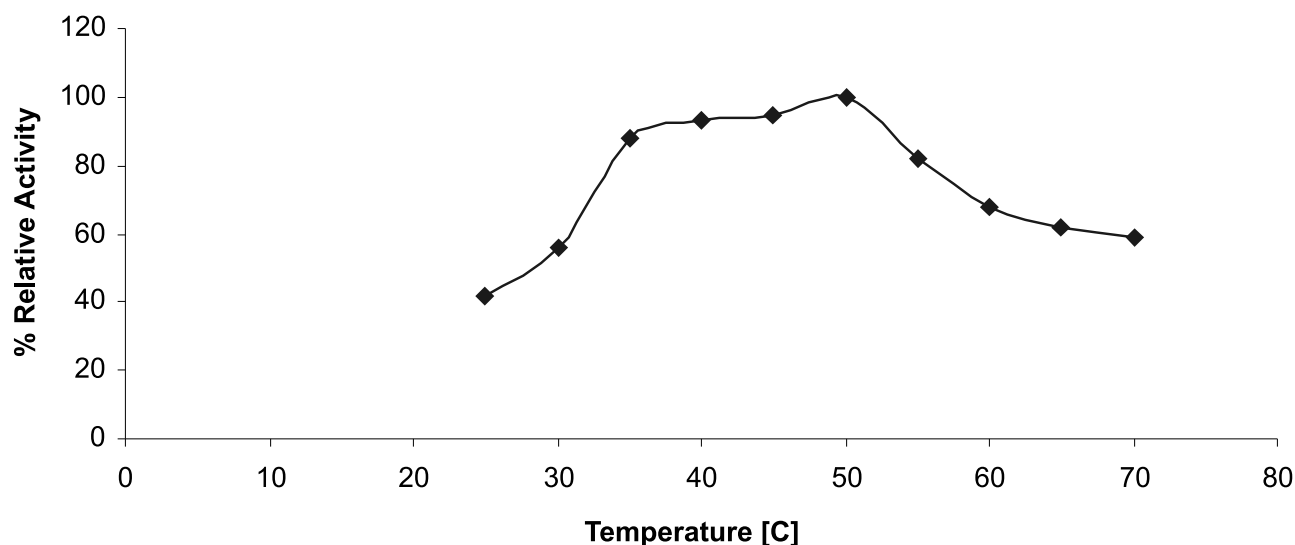


Figure 2. Effects of temperature on glucose isomerase activity of *Bacillus licheniformis*

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