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# Enhancement of Cyclodextrin Glucanotransferase Production by *Bacillus* G1 using Different Fermentation Modes

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**Abstract:** The objective of this study was to enhance the production of Cyclodextrin glucanotransferase (CGTase) produced by a local isolate *Bacillus* G1. CGTase produced in an optimized medium using different fermentation modes was investigated. The performance of batch mode, continuous mode and fed-batch mode was achieved in 5 L-stirred tank fermenter. Maximum CGTase production (77.49 U $mL^{-1}$) was achieved in fed-batch fermentation with 23.7% improvement compared to batch fermentation (62.63) and 16.6% improvement compared to continuous fermentation (66.47). CGTase productivity in fed-batch fermentation (1.6 U/mL/h) improved by 14.3% compared to that obtained by continuous fermentation (1.4 U/mL/h) and 23.1% improvement compared to batch fermentation (1.3 U/mL/h).

**Key words:** Cylodextrin glucanotransferase, *Bacillus* G1, batch fermentation, continuous fermentation, fed-batch fermentation

## INTRODUCTION

Cyclodextrin glucanotransferase (CGTase alpha-1, 4-glucan, 4-glycosytransferase, EC 2.4.1.19) converts starch and related α-1-4-glucans into cyclodextrin (CD) through an intramolecular transglycosylation reaction. Bacterial Glycosyltransferases are important carbohydrate converting enzymes involved in starch degradation, synthesis of exopolysaccharides and various glycosylated antibiotics. One example is cyclodextrin glycosyltransferase (CGTase); converting starch into cyclodextrins, which form stable inclusion complexes with many compounds. So, CGTase is an industrially important enzyme that produces cyclodextrin (CD). Cyclodextrin is a homogenous cyclic oligosaccharide with α-1-4 linkage usually composed of 6, 7, or 8 D-glycosyl residues known as α-CD, β-CD and γ-CD, respectively. It is a highly valuable modified starch. The potential application of CGTase in enzyme industry has increased impressively in recent years and its application is risen enormously with the increasing need of cyclodextrin in industry (Bender and Komiyama, 1978). Bacterial cyclodextrin glycosyltransferase represents one of the most important microbial amylolytic enzyme and is produced from various bacterial species mainly *Bacillus*. Certain species of Bacillus have been found to produce CGTases, which may differ from one another in various properties and especially in the ratio of cyclodextrin α:β:γ formed (Masao *et al*., 1986; Fujita *et al*., 1990). Due to the convenience of extraction and growth study, microbial CGTase has become increasingly important. Most of bacterial CGTase produced are mainly of α-β- and γ-CD, with six, seven, or eight glucose units respectively, via α (1-4-) glycoside linkage. CD is now used in food, pharmaceutical, chemical, cosmetic and agricultural industries because of their ability to form inclusion complexes with a variety of hydrophobic compounds by partial encapsulation them into their polar cavity, resulting in a prolonged stability and increased water solubility of these guest molecules (Tomita *et al*., 1993; Tonkova, 1998). CGTase is produced extracellulary by number of microorganisms, notably *B. stearothermophilius*, *B. ohbensis* (Jamuna *et al*., 1993) and *Bacillus* G1 (Wan *et al*., 2002). Various fermentation techniques such as submerged, solid state and surface culture; and mode of fermentor operations such as batch, fed-batch and continuous fermentation have been developed and used for improvement of various fermentation processes. Fermentation in most plants today takes place coincentily with the use of industrial enzymes, which are the major product of fermentation and the beer after fermentation is taken to the beer still. CGTase can be synthesized by *Bacillus* sp. either in submerged or solid-state cultivation system (Tonkova, 1998). For the overproduction of the

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enzyme, CGTase gene from various bacteria have been cloned (Palaheemo *et al*., 1992) but the protein yields reported for the recombinant *E.coli* or *B. subtilis* strain are not significantly high enough. The efficiency of *Bacillus* G1 to produce CGTase from tapioca starch on different scales and technique of fermentation has not yet been reported. However, information pertaining to the optimization of fermentation parameters for the maximization of enzyme synthesis is very scare (Jamuna *et al*., 1993; Tonkova, 1998). The major obstacle to the use of microbial enzyme for the manufacturing of bulk production is the production cost of the enzyme. Accordingly, research has been developed in many different areas aimed at reducing the operational cost of processes using the enzyme, for example genetic engineering technique which could increase the productivity of microorganism and thermal stability of an enzyme many fold. Enzyme immobilisation technique allows the enzyme to be separated physically from the mixture of substrate and product for re-use. Accordingly other methods which are more gentle and natural gets attention. Industrially, optimisation of the process conditions and fermentation processes with high product concentration, high yield and high production rates is preferred.

Very high gravity ethanol fermentation is one of process improvements for the ethanol production. It aims at increasing both ethanol concentration and fermentation rate. It can reduce capital costs. Energy costs per litre of alcohol as well as the risk of bacterial contamination (Narendranath and Power, 2005).

This study presents data from a study of batch, continuous and fed-batch fermentation to achieve high cell density and subsequently increase enzyme production.

## MATERIALS AND METHODS

**Chemicals:** All the chemicals used in this study are of analytical grade unless otherwise stated. Tapioca starch and peptone were purchased from BDH Chemical in 2002. Phenolphthalein was purchased from Merck.

**Strain:** The microorganism strain, *Bacillus* G1, was locally isolated from soil sample in Malaysia (Rosli *et al*., 2000). Morphological characteristics of this microorganism were identified according to the method described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

**Culture conditions:** The optimized medium, which reported by Ibrahim *et al*. (2005) was used in all the experiments. The media consist of (g $L^{-1}$): 40 tapioca starch, 20 peptone, 0.4 $MgSO_4.7H_2O$ and 10 $Na_2CO_3$. Gelatinized tapioca starch was prepared by heating the starch slurry to just above 59°C, which is the gelatinized temperature of tapioca starch (Swinkels, 1985). The temperature was set at 35°C and speed of agitation was at 150 rpm according to Ibrahim *et al*. (2003). Initial pH of the medium is of 9.98, aeration was maintained at 2 v/v/m. Samples were routinely taken at interval time.

**Fermenter and batch fermentation:** All the fermentation modes were performed using a 5L-stirred tank fermentor (B. Braun Biostat.) equipped with temperature and pH controller. Two six-bladed turbine impellers were used for agitation; a steam- sterilizable glass pH electrode was used to monitor the culture pH. Air was supplied into the culture broth through a single air sparger. The fermenter is provided with an outer jacket for water circulation. All of the fermentation modes were at working volume of 1 L. One hundred milliliters of preinoculum were grown in flask incubated in orbital-shaker (Gallenkamp UK) at 37°C and 200 rpm for sub-culturing 900 mL of media. The medium in the fermenter was sterilized at 121°C for 15 min.

**Continuous fermentation:** various flow rates 60, 120, 240, 360 and 420 mL $h^{-1}$ were studied to determine the optimum flow rate, using calibrated peristaltic pump. The reactor was operated at each flow rate for 24-48 h to ensure steady-state condition. Flow rate was monitored every 2 h by adjustment of the speed of the peristaltic pump manually when required. Continuous fermentation was initially run batch-wise for the first 24 h and then switched in a continuous mode for 144 h 20 (g $L^{-1}$) tapioca starch was fed continuously to the reactor with the optimum flow rate obtained. The level of the culture broth in the reactor was kept constant at 1 L. Samples were collected in duplicate at each dilution rate at steady-state conditions and analyzed for enzyme activity.

**Fed-batch fermentation:** Fed-batch fermentation was run batch-wise for the first 24 h and switched in a fed-batch mode for 144 h. Two hundred milliliter of 20 (g $L^{-1}$) tapioca starch was added intermittently at one-day interval for 5 days through the bottom by a peristaltic pump i.e. gelatinized tapioca starch was added 5 times during the fermentation and the eluant was collected, before and after each cycle, from the outlet provided at the top of the reactor. Fed-batch fermentation was stop when the volume of the media reaches 2 L. The speed of agitation was at 300 rpm.

**Analytical methods:** During the fermentation experiments, 20 mL samples were collected at time interval for chemical analysis. The sample was centrifuged at 30,000 rpm for 15 min in 4°C. The supernatant was used to determined the CGTase activity (Kaneko *et al.*, 1987) and total carbohydrate concentration (Dubois *et al.*, 1956). While the pellet was washed with buffer in order to remove the starchy materials attached to the pellet. The suspensions free from starchy materials were filtered using a pre-weight filter paper and dried in an oven at 60°C until constant weight for measurement of dry weight (g $L^{-1}$). The enzyme activity (U) was expressed in terms of μmole of β-cyclodextrin released per mL in one minute.

The following simplified batch fermentation kinetics models for cell growth and product formation were used to evaluate the kinetics of cgtase by *Bacillus* G1. The growth yield (Y) may be estimated from the amount of a substrate utilized (ΔS) or the amount of product formed (ΔP). Productivity is amount of enzyme produced at particular time (g/L/h) (Pirt, 1975).

## RESULTS

The bacterium in this study was identified as *Bacillus* sp. of gram +ve, spore forming, short rod-shape and motile. It grew well in alkaline media of pH from 8.0 to 10.3.

**Batch culture:** The typical time course of CGTase production by *Bacillus* G1 in 5 L stirred tank fermenter using tapioca starch as a carbon source is shown in Fig. 1. It is clear that CGTase production of (62.63 U $mL^{-1}$) and biomass concentration of (15.94 g $L^{-1}$) were reached maximum at 12 h after inoculation and plateau off for the next two days. Enzyme production was found to be concomitant with growth.

**Continuous fermentation:** Table 1 shows the enzyme activity plotted against different dilution rates. It can be seen that the maximum activity of (59.65 U $mL^{-1}$) occurred at dilution rate of 0.36 $h^{-1}$ and decreased as the dilution rate increased.

Enzyme productivity which represents the efficiency of the system (Abdel-Naby *et al.*, 2000) was calculated by multiplying the dilution rate by the enzyme activity. Enzyme productivity increased with increasing the dilution rate ups to 0.36 h and decrease thereafter (Table 1).

**Fed-batch fermentation:** Figure 2 shows the time course of fed-batch fermentation of CGTase. It is clear that at

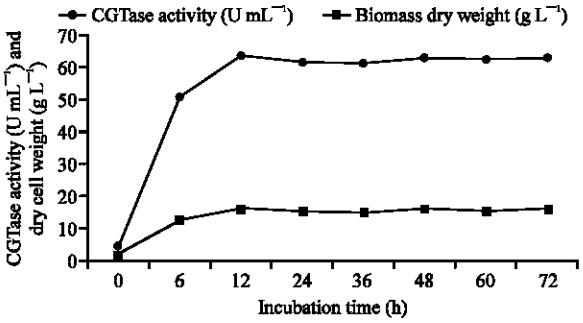


Fig. 1: Time course of CGTase production by *Bacillus* G1 in stirred tank fermenter in optimized medium, temperature of 35°C, agitation of 150 rpm and 1 L medium volume

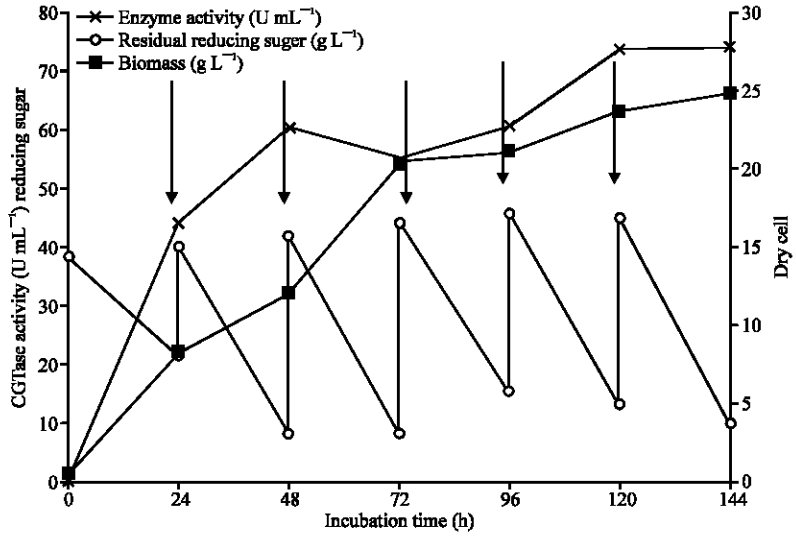


Fig. 2: Time course of cgtase production in fed-batch fermentation. Arrows indicate the time when tapioca starch is added. Culture conditions: temperature of 35°C, agitation of 300 rpm and 2 v/v/m

Table 1: Effect of dilution rate on the enzyme productivity

| Dilution rate ($h^{-1}$) | Enzyme activity (U $mL^{-1}$) | Enzyme productivity (U mL/h) |
|---|---|---|
| 0.06 | 37.93 | 2.276 |
| 0.12 | 39.40 | 4.728 |
| 0.24 | 45.98 | 11.035 |
| 0.36 | 59.65 | 21.474 |
| 0.42 | 33.43 | 14.041 |

Table 2: Kinetic comparison of CGTase activity in different fermentation modes

| System | Pm (U $mL^{-1}$) | Xm (g $L^{-1}$) | Productivity (U $h^{-1}$) | Yp/x (μmol $mg^{-1}$) |
|---|---|---|---|---|
| Batch fermentation | 62.63 | 15.94 | 1.3 | 3.9 |
| Fed- batch fermentation | 77.49 | 21.98 | 1.6 | 3.5 |
| Continuous fermentation | 66.47 | 19.72 | 1.4 | 3.4 |

Pm: Maximum CGTase concentration during fermentation, Xm: Maximum cell concentration, P: Enzyme productivity at 48 h, Yp/x: yield of CGTase based on biomass

120 h the activity and biomass reached maximum (77.49 U $mL^{-1}$) and (21.09 g $L^{-1}$), respectively. The growth profile of *Bacillus* G1 in fed-batch fermentation was 1.3 times more than that in batch fermentation (15.94 g $L^{-1}$). The maximum CGTase production in Fed-batch mode (77.49 U $mL^{-1}$) was increased about 1.2-fold than that obtained in batch Fermentation (62.63 U $mL^{-1}$). The enzyme activity of 5 cycles was remaining between 64.18 and 77.49 U $mL^{-1}$ throughout the cycles. It has been shown that *Bacillus* G1 was able to synthesize CGTase consistently. In all cases, CGTase production increased linearly with time.

The conditions in fermenter resulted in a high growth rate, high biomass concentration and then high CGTase activity achieved. CGTase production in fed- batch fermentation was increased 23.7% improvement compared to batch fermentation. And 16.6% compared to continuous fermentation.

**Comparison studies between the three modes of cultivation:** The objectives of this comparison are to study the influence of mode of fermentation of CGTase produced by *Bacillus* G1 and to evaluate the growth kinetics of *Bacillus* G1 under various fermentation conditions in term of yield p/x and productivity.

Table 2 shows that the highest productivity was obtained in fed-batch fermentation of 1.6 (U $h^{-1}$). The yields p/x in all fermentation modes were slightly similar.

Table 3 shows the volumetric rate of enzyme formation ($Q_p$) and cell mass concentration ($Q_x$) in the culture at a particular time. It appeared that maximum in $Q_p$ and $Q_x$ are obtained in fed-batch fermentation and $Q_x$ is lower because *Bacillus* G1 entered the stationary phase earlier as CGTase production is high.

Table 3: Kinetic of growth of *Bacillus* G1

| Fermentation mode | $Q_p$ | $Q_x$ |
|---|---|---|
| Batch fermentation | 1304.8 | 0.332 |
| Fed-batch fermentation | 1614.4 | 0.458 |
| Continuous fermentation | 1384.8 | 0.411 |

$Q_p$ is the rate of enzyme produced in the culture media at particular time, g enzyme produced /L/h. $Q_x$ is the rate of gram cell mass formation at time, g cell mass formation /L/h (Pirt, 1975)

## DISCUSSION

There has been a continuing interest in cyclodextrin glucanotransferase due to it's prime role in the production of an industrial product cyclodextrin. The application of cyclodextrin is the molecular chelating agents in pharmaceutical, food processing, cosmetics and agricultural chemicals (Szetjli, 1998).

*Bacillus* G1 CGTase can be successfully produced from optimised media in batch culture in shake-flask (Ibrahim *et al*., 2005). To ascertain further the growth behaviour and enzyme synthesis pattern of the culture, batch and continuous fermentation in stirred tank fermenter has been proposed. As CGTase produced in the presence of the substrate so tapioca starch was fed to the reactor.

Bacteria growth in batch shake- flask (11.95 g $L^{-1}$) (Ibrahim *et al*., 2005) and in batch 5-l fermenter in this study is (15.94 g $L^{-1}$) where enzyme activity in batch shake flask is (51.54 μ $mL^{-1}$) and in batch fermenter is (62.63 μ $mL^{-1}$) the reason of these changes in the levels may be due to the different in hydrodynamic conditions and shear rate created in shake-flask. In addition, fermentation in shake-flask is subjected to oxygen limitation.

The performance of CGTase fermentation by *bacillus* G1 was improved when the fermentation was carried out in five litre stirred tank fermenter using continuous operation (66.47 μ $mL^{-1}$) compared to batch fermentation in the fermenter (62.63 μ $mL^{-1}$). Little improvement of CGTase production in continuous fermentation of tapioca starch, may be due to high viscosity of starch during the initial stages of fermentation resulted in imperfect mixing and insufficient oxygen supply during fermentation of high tapioca starch concentration so the optimum aeration conditions for CGTase production were not achieved in continuous fermentation, or may be due to the high viscosity of the medium that caused improper mixing and reduced mass transfer rate.

High enzyme activity and productivity obtained in continuous fermentation may be due to the fact that the total amount of enzyme output from the reactor increases, although the activity is lower, because of high flow rate. But after a particular dilution rate, the output of the enzyme decreases because of high flow rates and low

enzyme yield. This implies that in operating a continuous reactor, there may be an optimum dilution rate at which maximum enzyme productivity is attained. Similar behavior in the immobilized packed-bed reactor was reported (Ramakrishna *et al*., 1994). Lane and Pirt (1973) produced high CGTase activity at low dilution rates of 0.03 to 0.05 $h^{-1}$, but the residual starch was present at higher dilution rates and CGTase synthesis was repressed. This different in dilution rate due to that, in their experiment, CGTase produced when the growth was ceased. This result also agreed with the finding of Jamuna and Ramakrishna (1992) in the similar behavior of α-amylase production in continuous culture by *Bacillus cereus* that α-amylase productivity increased with increase in dilution rate up to 2.85 $h^{-1}$ and decreased thereafter. Yun *et al*. (1994) obtained higher isomalto-oligosaccharides at a particular dilution rate of 0.1 $h^{-1}$ at 50°C using 60% maltose by *Aureobasidium pullulans*. Continuous culture was investigated in packed-bed reactor for CGTase biosynthesis, maximal productivity of 23 KU/L/hl with enzyme concentration of 48.0 U $mL^{-1}$ was attained at dilution rate of 0.48 $h^{-1}$ and maximum productivity of 30.23 KU/L/h with enzyme concentration of 53.0 U $mL^{-1}$ at dilution rate of 0.57 $h^{-1}$ in fluidized-bed reactor (Abdel-Naby *et al*., 2000). CGTase was produced stable from *Bacillus cereus* in chemostat culture over a period of 400 h with maximum productivity of 5.4 KU/L/h. Three-fold higher than obtained in batch culture 1.75 KU/L/h at 0.06 $h^{-1}$ dilution rate (Jamuna *et al*., 1993).

The time course of CGTase production and cell concentration from *Bacillus* G1 in continuous fermentation was studied at a dilution rate of 0.36 ($h^{-1}$). Figure 3 shows that cell concentration of *Bacillus* G1 in continuous fermentation increased to 19.72 g $L^{-1}$ compared to 15.94 g $L^{-1}$ in batch fermentation, with 1.2-fold and 23.7% improvement. *Bacillus* G1 CGTase production was improved by 6.13% in continuous fermentation (66.47 U $mL^{-1}$) compared to batch fermentation in agitated vessel (62.63 U $mL^{-1}$). Lane and Pirt (1973) obtained CGTase in chemostat culture at D = 0.03 $h^{-1}$ using defined medium containing 13 g starch/L were 2.75 times greater than the maximum obtained by batch cultivation and about 20 times greater than other reports using medium containing diced potato.

Further improvement in the production of *Bacillus* G1 CGTase was achieved in Fed-batch fermentation using tapioca starch as substrate between 64.18 U $mL^{-1}$ and 77.49 U $mL^{-1}$ throughout the cycles, CGTase production increased linearly with time. Jun *et al*. (2009) stated that Fed-batch cultures with suspended cells revealed that 1,3-PD production was more effective when utilizing raw

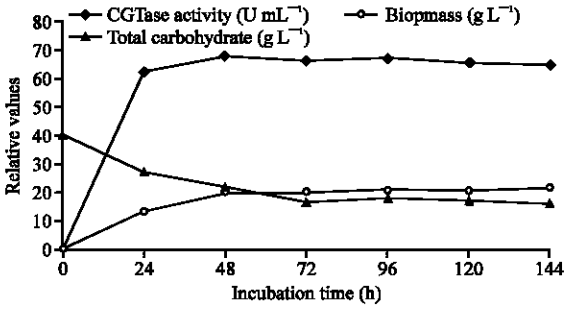


Fig. 3: Time course for the production of CGTase from *Bacillus* G1 in continuous fermentation at 0.36 $h^{-1}$ dilution rates, temperature of 35°C and agitation of 150 rpm and aeration of 2 v/v/m

glycerol than pure glycerol (productivity after 47 h of fermentation, 0.84 g/L/h versus 1.51 g/L/h with pure and raw glycerol, respectively), he also mentioned that repeated fed batch with immobilized cells is an efficient fermentor configuration and raw glycerol can be utilized to produce 1,3-PD without inhibitory effects caused by accumulated impurities. Park *et al*. (1997) used optimized fed-batch fermentation of recombinant *Escherichia coli*, resulting in a maximum CGTase activity of 62.9 U $mL^{-1}$ and a final cell mass of 53.5 g $L^{-1}$, corresponding to a 31-fold increase in CGTase activity and a 29-fold increase in cell mass compared with the control batch fermentation. Continuous culture studies of amylase and protease synthesis by *B. amyloliquefaciens* and some strain of *B. subtiliis* have, after initially showing high enzyme level in batch culture, often been followed by fluctuating enzyme yield which subsequently decline during continuous culture. This pattern of enzyme yield can be explained by the occurrence of low-producing or non-producing mutants (Fencl and Pazlarova, 1982). Heyland *et al*. (2010) used $^{13}C$-tracer based metabolic flux analysis in batch and fed-batch experiments. At a controlled growth rate of 0.12 $h^{-1}$ in fed-batch experiments an increased TCA cycle flux of 1.1 mmol/g/h compared to 0.7 mmol/g/h for the recombinant and reference strains, respectively.

To compare between the three modes of cultivation, The yields p/x in all fermentation modes were slightly similar. this mat is due to that *Bacillus* G1 CGTase is growth-associated type. The fed-batch culture in this study is a satisfactory mode of operation of the reactor, since it allowed the overall biomass yield to be increased significantly and subsequently CGTase activity. Fed-batch culture is an ideal method for high *Bacillus* G1 CGTase production (77.49 U $mL^{-1}$).

## CONCLUSION

Fed-batch fermentation is an appropriate potential technique for *Bacillus* G1 CGTase production that gave maximum activity (77.49 U $mL^{-1}$) compared to batch fermentation (62.63 U $mL^{-1}$) and continuous fermentation (66.47 U $mL^{-1}$) in 5 L-stirred tank fermenter.

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