Nepal J Biotechnol. 2024 Dec; 12 (2):116-120 Research Article DOI: https://doi.org/10.54796/njb.v12i2.340

Nepal Journal of Biotechnology
Publisher: Biotechnology Society of Nepal ISSN (Online): 2467-9313
Journal Homepage: https://nepjb.com/index.php/NJB ISSN (Print): 2091-1130

Effect of Different Carbon and Nitrogen Sources on the Activity of Cycloamylase (Cyclomaltodextrin Glucanotransferase) Produced by *Sutclifella cohnii*

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Received: 14 Nov 2024; Revised: 29 Nov 2024; Accepted: 07 Dec 2024; Published: 31 Dec 2024

Abstract

Cyclomaltodextrin glucanotransferase (CGTase) is an important group of microbial amylolytic enzyme that helps in the conversion of starch and other polysaccharides into cyclic oligosaccharides. This study was carried out to determine the effect of alternative carbon and nitrogen sources on the production of cyclomaltodextrin glucanotransferase from *Sutclifella cohni*. Rice- soybean water (RS), yam-soybean water (YS), rice-fish water (RF), *fufu*-fish water (FuF), fufu- soybean water (FuS), rice-meat water (RM), yam-meat water (YM), *fufu*-meat water (FuM), and yam-fish water (YF) were used as substitute for soluble potato starch in the production medium.. The different carbon and nitrogen sources studied supported the growth and production of CGTase. The optimal pH and temperature was 8.0 and 45°C, respectively. The lowest K_m (4.17 mg/mL) was observed with RM as the growth medium. This study concludes that food waste could be used as substitute for soluble starch in the production of CGTase, for industrial application.

Keywords: Biotechnology, Carbon, Cyclomaltodextrin glucanotransferase, Industrial, Nitrogen.

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Introduction

Microbes have a tremendous capacity in nature to create a variety of enzymes, which have been utilized commercially over time. The foundation for industrial enzyme production has been microbial enzymes. Exploring the extracellular enzymatic activity in various microbes isolated from various habitats has sparked attention due to the innovation of utilizing microbes as sources of enzymes for industrial and biotechnological applications [1, 2]. Hydrolases are a group of hydrolytic enzymes frequently employed as biochemical catalysts and utilize water as a source of hydroxyl groups while breaking down substrates. In the Carbohydrate-Active Enzymes database (CAZy, www.cazy.org), Family 13 of the glycoside hydrolases (GH) is currently a significant group. The subfamily GH13 can hydrolyze at least two of the three substrates, Cyclomaltodextrin (CDs), pullulan, and starch [3] because it typically possesses the N-terminal starch-binding domain (SBD), which is categorized as the carbohydrate-binding module family CBM34 [4]. Cyclomaltodextrin glucanotransferase (cycloamylase, CGTase) is an amylolytic enzyme capable of converting starch and other related polysaccharides into cyclic oligosaccharides called cyclodextrins due to its unique shape [5].

The cost of enzyme production keeps rising and carbon sources alone accounts for up to 50% of these costs [6]. It is also well known that the price of the growing medium is thought to account for 30–40% of the cost of manufacturing industrial enzymes. This worrisome trend has made industrial scientists take interest in the search and development of alternative carbon and nitrogen sources that could replace the conventional ones used in industries. Therefore, it is important to make use of low-cost carbon sources to increase enzyme content and productivity. Less expensive carbon sources such as date syrup [7], *mahuva* flower extract [8], corn steep liquor [9], biowaste [10], jackfruit seed hydrolysate [11] have been used as a carbon source for enzyme production by *Bacillus* spp. The expensive cost of the enzyme is the main barrier to its large-scale use in industry [12]. This has prompted researchers to seek for more affordable alternatives to make this enzyme. Researchers have studied the responses of various bacteria sources of amylase enzymes to various sources of carbon and nitrogen over time. However, neither the mechanism nor the influence of carbon and nitrogen sources on the production of the enzyme cyclomaltodextrin glucanotransferase by *S. cohnii* is known. This work

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therefore, aims to study the effect of different carbon and nitrogen sources on the production of cyclomaltodextrin glucanotransferase by *S. cohnii*.

Materials and Methods

S. cohni (inoculum) was obtained from the Department of Microbiology, Federal University of Technology, Minna, Niger State., Carbon sources (Rice water, Yam water, Fufu water), Nitrogen sources (fish water, meat water, soya beans) was obtained from wastes of common food wastes. All reagents used were of analytical grade and was obtained from reputable chemical firms. **Table 1** shows the formulation of the different carbon (C) and nitrogen (N) sources in equal ratios (50:50)

Table 1. Formulation of carbon and nitrogen sources

C-N	Ratio (1:1)
RS	Rice water and soyabean water
RF	Rice water and fufu water
RM	Rice water and meat water
FuS	Fufu water and soyabean water
FuF	Fufu water and fish water
FuM	Fufu water and meat water
YS	Yam water and soyabean water
YF	Yam water and fish water
YM	Yam water and meat water

Preparation of bacterial inoculum

The selected bacterial strain was cultured in 50 mL of alkaline Horikoshi (II) medium (pH 10.5), which served as the basal medium. The medium composition included 1 % soluble starch, 0.5 % peptone, 0.5 % yeast extract, 0.1 % KH_2PO_4, 0.02 % $MgSO_4 \cdot 7H_2O$, and 1 % Na_2CO_3. The culture was incubated at 37°C for 24 hours on an orbital shaker set to 120 rpm. After incubation, the cells were harvested by centrifugation at 5,000 rpm for 15 minutes, washed with normal saline (0.85% w/v), and resuspended in normal saline to achieve an optical density of 0.05 at 660 nm, which was used as the inoculum [13].

Production of CGTase

Cyclomaltodextrin glucanotransferase (CGTase) was produced under anaerobic conditions in 1 L conical flasks containing 150 mL medium. The medium contained RS, RF, RM, FuS, FuF, FuM, YS, YF and YM each containing 5 g/L $MgSO_4.7H_2O$, 1 g/L K_2HPO_4 and 10 g/L Na_2CO_3 at a pH of 7.5. After 30 h of growth at 65 °C, bacterial cells were removed from the growth medium by centrifugation at 10, 000 g for 15 min. Cell-free supernatant was used as the crude enzyme [13].

CGTase assay

Cycloamylase activity was assessed using a modified phenolphthalein assay method. The reaction mixture consisted of 1% soluble starch in 50 mM Tris-HCl buffer (pH 10) and 1 mL of crude enzyme. This mixture was incubated at 37°C for 15 minutes. The reaction was terminated by placing the mixture in a boiling water bath for 3 minutes. Subsequently, 4 mL of 0.04 mM phenolphthalein dissolved in 125 mM Na_2CO_3-$NaHCO_3$ buffer was added. The absorbance was measured at 550 nm [14]. The amount of β-cyclodextrin produced was quantified using a standard curve constructed with β-cyclodextrin concentrations ranging from 0 to 500 µg/mL. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of β-cyclodextrin per minute under the specified conditions. [13].

Determination of Growth Kinetics

The growth kinetics for the bacterial culture was studied by inoculating the alkaline Horikoshi (II) medium (basal medium), pH 10.5, with the different formulations of carbon and nitrogen sources. The fermentation media were incubated at 37 °C on an incubator shaker at 120 rpm. Aliquots of 2 mL were withdrawn every 2 h for 96 h. The optical density was determined at 660 nm and the supernatant obtained after centrifugation at 10,000 g for 15 min was assayed for CGTase activity [13].

Effect of pH on CGTase production

1 % suspensions of the carbon and nitrogen sources in 0.1 M Glycine-NaOH buffer pH 6.5-9.0 were gelatinized at 70 °C for 1 h. One gram of solubilized starch was reacted with 12 U of the enzyme at 50 °C [14].

Effect of Temperature on CGTase production

The effect of temperature on production of the enzyme was investigated by varying the temperature in the range of 25- 50°C [13].

Determination of kinetic parameters

The Michaelis constant (K_m) and the maximum rate (V_{max}) of the enzyme were calculated according to the method described by [15]. Various concentration of the C-N formulations in 50 mM Tris HCl buffer (pH 8.0) was taken and enzyme activity was assayed as described above.

Results

The effect of nine different nitrogenous compounds on the growth of *S. cohnii* was determined and shown in **Figure 1**. All the carbon and nitrogen sources caused an increase in the bacterial growth rate at 0 hr to 12 hr. This

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increase in growth rate continued up until 24 hr for RS, RF and YF before a decrease occurred at 30 hr. In contrast, RM, FUS, FUM, YS, YM, FUF caused a decrease at 18 h and at 30 h.

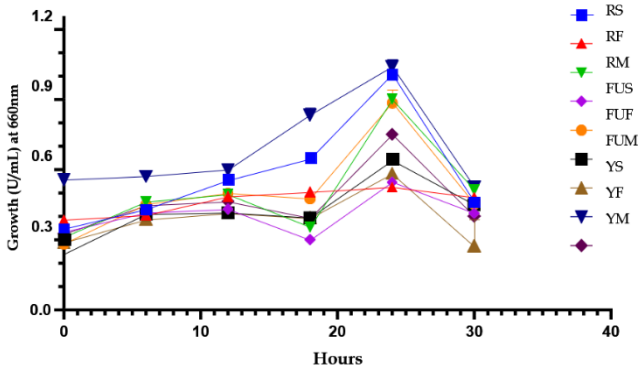

Figure 1. Effect of different carbon and nitrogen sources on bacteria growth.**Key**: RS- Rice water and soyabean water; RF: Rice water and fufu water; RM: Rice water and meat water; FuS- Fufu water and soyabean water; FuF- Fufu water and fish water; FuM- Fufu water and meat water; YS- Yam water and soyabean water; YF-Yam water and fish water; YM- Yam water and meat water.

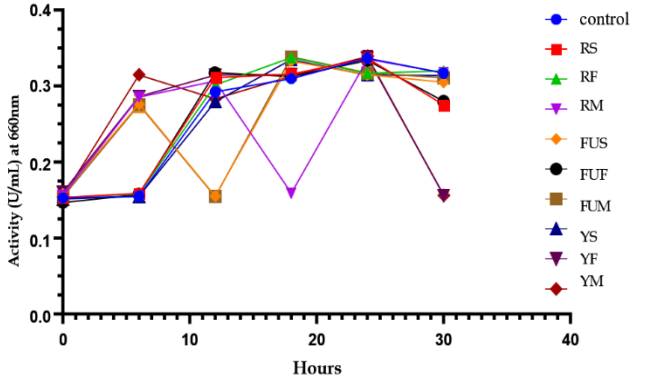

Figure 2. Effect of different carbon and nitrogen sources on CGTase production. **Key**: RS- Rice water and soyabean water; RF: Rice water and fufu water; RM: Rice water and meat water; FuS- Fufu water and soyabean water; FuF- Fufu water and fish water; FuM- Fufu water and meat water; YS- Yam water and soyabean water; YF-Yam water and fish water; YM- Yam water and meat water.

Effect of different carbon and nitrogen sources on the production of CGTase

The effect of nine different nitrogenous compounds on the production of cycloamylase enzyme was determined and shown in **Figure 2**. The activities of the control (normal media) steadily increased from 0 hr to 24 hr before a decrease occurred at 30 hr. All the carbon and nitrogen sources caused an increase in enzyme production from 0 hr to 6 hr. For RS and YF, the increase continued up until 24 hr before a decrease occurred at 30 hr. FUS, YS and YF caused an increase in cycloamylase activity up to 18 h before a decrease set in at 24 hr then an increase again at 30 hr. RM and FuF caused an increase up until 12 hr, a decrease occurred at 18 hr and at 30 hr. FM caused a fluctuating decrease in activity at 12 hr, 24 hr and this continued till the 30 hr. YM caused a decrease in activity at 12 hr, increase at 18 hr, 24 hr before falling at 30 h.

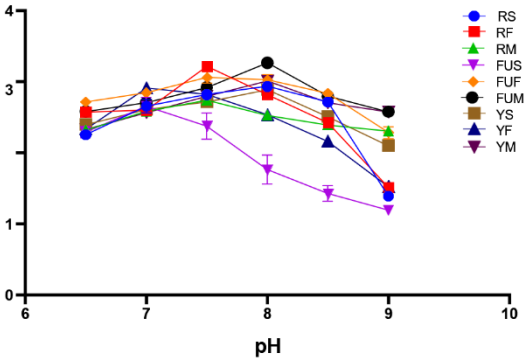

Figure 3. Effect of C-M sources on Cyclomaltodextrin glucanotransferase production at different pH. **Key**: RS- Rice water and soyabean water; RF: Rice water and fufu water; RM: Rice water and meat water; FuS- Fufu water and soyabean water; FuF- Fufu water and fish water; FuM- Fufu water and meat water; YS- Yam water and soyabean water; YF-Yam water and fish water; YM- Yam water and meat water.

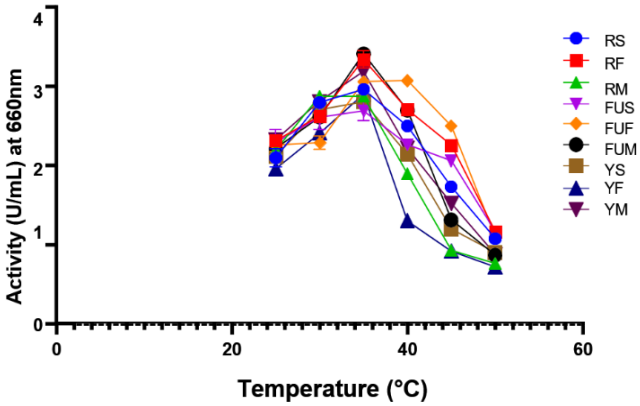

Figure 4. Effect of C-M sources on Cyclomaltodextrin glucanotransferase production at different temperature **Key**: RS- Rice water and soyabean water; RF: Rice water and fufu water; RM: Rice water and meat water; FuS- Fufu water and soyabean water; FuF- Fufu water and fish water; FuM- Fufu water and meat water; YS- Yam water and soyabean water; YF-Yam water and fish water; YM- Yam water and meat water.

Effect of C-N media on CGTase production at different pH

The effect of the different carbon and nitrogen sources on the production of Cyclomaltodextrin glucanotransferase was determined at different pH as shown in **Figure 3**. The results showed the steady increase from pH 6.5 to 8.0 for RS, YS, YM and FuM before a decline set in at pH 8.5 to 9.0. On the other hand, the enzyme production rose with RF, RM and FuF from pH 6.5 to 7.5 before a reduction in production set in at pH 8.0 and continued up till pH 9.0. The production with YF and FuS increased at pH 6.5 to 7.0 with a decline setting in at pH 8.0 to 9.0

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Effect of C-N media on CGTase production at different temperature

The effect of the different carbon and nitrogen sources on the activity of cycloamylase enzyme was determined at different temperature as shown in **Figure 4**. The results showed that activity increased from 25 °C to 35 °C and started declining at 40 °C to 50 °C.

Kinetic constants on cyclomaltodextrin glucanotransferase using different C-M media

The kinetic parameters (K_m and V_{max}) obtained from Cyclomaltodextrin glucanotransferase producing bacteria (*S. cohni*) using different carbon and nitrogen sources are presented in **Table 2**.

Table 2. Kinetic parameters of carbon and nitrogen sources

Substrate	K_m (mg/mL)	V_{max} (µmol/min)
RS	9.09	2.95
RF	11.24	3.31
RM	4.17	2.67
FuS	12.17	2.91
FuF	17.41	3.56
FuM	11.90	3.17
YS	7.70	2.87
YF	7.22	3.01
YM	13.42	3.12

Discussion

Fermentation medium cost is one of the important factors in industrial and microbial enzyme production and utilization of agro-industrial and domestic food waste can play a vital role in the reduction of this cost. Locally-isolated strains and cheap substrates can produce inexpensive amylase and reduce the enzyme's production cost [16]. Cyclodextrin glucanotransferase also known as cycloamylase is an example of a thermostable amylase [17]. The effect of various carbon and nitrogen sources on the growth and production of the enzyme was determined and its effects on temperature and pH. Fermentation conditions of *Sutclifella cohni* were optimized in terms of carbon and nitrogen source and influence of pH to enhanced growth and the highest CGTase activity. All the carbon and nitrogen sources showed an increase in the bacterial growth rate at 0 hr to 12 hr. This increase in growth rate continued up until 24 hr for RS, RF and YF before a decrease occurred at 30 hr. In contrast, RM, FUS, FUM, YS, YM, FUF which showed a decrease at 18 hr and at 30 hr. Similarly, the results in **Figure 2** indicated activities of the control (normal media) steadily increased from 0 hr to 24 hr before a decrease occurred at 30 hr. Similarly, the carbon and nitrogen sources caused an increase in cycloamylase production at 0 hr to 6 hr. For RS and YF, the increase continued up until 24 hr before a decrease occurred at 30 hr. FUS, YS and YF showed an increase in the enzyme production up to 18 h before a decrease set in at 24 hr then an increased again at 30hr. RM and FuF showed an increase up until 12 hr, a decrease occurred at 18 hr and at 30 hr. FuM showed a fluctuating decrease in activity at 12hr, 24 hr and this continued till the 30 hr. YM showed a decrease in production at 12 hr, increase at 18 hr, 24 hr before falling at 30 hr. This study indicated that the enzyme production peaked after 24 hr when RS, RM, FUF, YF and YM were used. The findings of this study indicate that cycloamylase production is growth-dependent. The observed decline in enzyme activity with prolonged incubation may be attributed to reduced cell growth, nutrient depletion, and changes in the final pH [18]. Previous research on amylase production in solid-state fermentation has shown similar trends. For instance, *Bacillus licheniformis* exhibited peak amylase production at 36 hours [19], while *Bacillus amyloliquefaciens* achieved maximum activity after 42 hr when supplemented with wheat bran and groundnut oil cake as carbon sources [20]. Under submerged fermentation conditions, *Bacillus amyloliquefaciens* reached optimal amylase production at 48 hours [21]. Additionally, *Bacillus licheniformis* yielded the highest amylase levels after 72 hr in shaker flask conditions [22].

The influence of pH on the activity of the enzyme produced using different carbon and nitrogen media showed significant changes in enzyme activity as the pH changes, and the maximum yield was found at the optimal pH of 8.0 (RS, RF, RM and FUM) while the least optimal pH was obtained at 7.0 with FuS. Amylase production from *Bacillus cereus* under solid-state fermentation was reported in alkaline pH [9]. However, [23] obtained maximum amylase concentration at acidic pH of 6.00 from *Penicillium chrysogenum*. Thermo-alkaliphilic microbial strains are considered as industrially-important strains due to their commercial value, especially alkaline pH-stable amylase, which could be used in detergent formulations.

The effect of pH on enzyme activity was evaluated using various carbon and nitrogen media, revealing significant variations with pH changes. The highest enzyme yield was observed at an optimal pH of 8.0 (RS, RF, RM, and FUM), while the lowest yield occurred at pH 7.0 with FuS. Amylase production by *Bacillus cereus* under solid-state fermentation has been reported to occur in alkaline pH conditions [9]. In contrast, Halder *et al*. [23] reported maximum amylase production at an acidic pH of 6.0 from *Penicillium chrysogenum*. Thermo-alkaliphilic microbial

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strains are particularly valuable for industrial applications due to their ability to produce alkaline pH-stable amylase, which is especially useful in detergent formulations.

The results in **Table 2** showed the kinetic parameters of CGTase produced by *S. cohni* using different carbon and nitrogen media. The enzyme had the highest K_m and V_{max} values of 17.54 mg/mL and 3.56 µmol/min with FuF as inducer. However, the lowest K_m value of 4.17 mg/mL was achieved using RM as the inducer. K_m is a measure of the affinity an enzyme has for its substrate. A low K_m indicates a high binding of the enzyme to the substrate, while a high K_m indicates low affinity of the enzyme to the substrate.

The result of this study showed that the alternative carbon and nitrogen sources improved the growth of the bacteria and supported the production of Cyclomaltodextrin glucanotransferase (cycloamylase) at a pH of 8.0 and optimal temperature of 35 ^oC.

Conclusion

It is therefore safe to conclude that the bacteria *S. cohnii* has the ability to utilize the various carbon and nitrogen sources from food waste for growth as well as for the production of cycloamylase. These alternative carbon and nitrogen sources could serve as alternative replacement of the growth media industrial production of cycloamylase.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors Contribution

Conceptualization- OSA, ECE; Methodology, ECE, OSA, AYK, OAO; Data curation- OSA, ZA; Writing (Original draft)- OSA, ZA; Writing (review and editing)- OSA, ECE, AYK, OAO; Supervision: ECE, AYK, OAO. All authors have read and agreed to the published version of the manuscript.

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