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# Effect of Nitrogen Sources on *Penicillium chrysogenum* Biomass and Amylase Production Using Cassava Whey

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**Abstract:** Industrial wastes like starchy wastes cause environmental pollution especially of waterways however, they can be used for biosynthesis of enzymes (amylases) thereby reducing their pollution impact on the environment. Amylases are starch degrading enzymes that have great importance in biotechnology and starch processing industries. This study aimed at optimizing *Penicillium chrysogenum* biomass and amylase production in cassava whey media using organic and inorganic nitrogen supplementations. Biomass yield of *P. chrysogenum*, amylase activity, reducing sugar, titratable acidity and pH values were determined using standard techniques during submerged fermentation with cassava whey. The highest *P. chrysogenum* biomass (5.0 ± 0.02 g/L) yield and amylase production (8.0 ± 0.04 mU/g) were obtained with ammonium nitrate ($NH_4NO_3$) at 8 d while the least biomass and amylase yield 3.0 ± 0.40 g/L and 1.71 ± 0.01 mU/g respectively were obtained from the unsupplemented media. The present study showed that cassava whey, a cheap agro-waste may be a good substrate for amylase production when supplemented with ammonium nitrate using *P. chrysogenum*

**Keywords**: Cassava whey, Amylase, reducing sugar, *Penicillium chrysogenum*

## Introduction

Disposal of industrial wastes poses environmental problems especially in waterways due to obnoxious odor and high organic matter content that may lead to high biochemical oxygen demand (BOD) and high chemical oxygen demand (COD) in these waterways (Ruban *et al*., 2013). However, their utilization as renewable resources are of utmost importance for biosynthesis of enzymes (amylases) which could also reduce their pollution impact on the environment (Krishma and Radhathirumalaiarasu, 2017; Ayansina *et al*., 2017; Oshoma *et al*., 2017). Low cost agricultural wastes like cassava whey (due to high organic matter such as CHO content) are suitable substrate for the growth of microorganisms and variety of metabolites production like enzymes and ethanol (Goyal *et al*., 2005; Krishma and Radhathirumalaiarasu, 2017; Oshoma *et al*., 2017)

In recent years, the potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Pandey *et al*., 2000; Abu *et al*., 2005; Oshoma *et al*., 2017). The major advantage of using microorganism for amylase production (especially fungal amylases) is due to their high accepted generally recognized as safe (GRAS ) status, productivity, thermostability, their extracellular means and low cost of production (Aiyer, 2005; Saleh *et al*., 2011; Ayansina *et al*., 2017). Amylases are enzymes that break down starch into simple sugars (Pandey *et al*., 2000; Oshoma *et al*., 2017). These enzymes have received a great deal of attention because of their applications in the food, brewing, textile, detergent and pharmaceutical industries, and currently comprise about 30 % of the world's enzyme production (Demirijan *et al*., 2001; Gupta *et al*., 2003, 2008; Tanyildizi, 2005; Prakash and Jaiswal, 2009; Krishma and Radhathirumalaiarasu, 2017).

Many fungi have been found to be good sources of amylolytic enzymes, therefore, screening for effective and efficient fungal isolates for amylase production will reduce production cost in industries which employ amylase and provide economic opportunities (Omemu *et al*., 2005; Ayansina *et al*., 2017). Penicillium species are among some of the fungi that produce extracellular amylases of commercial value (Ayansina *et al*., 2017). They are ubiquitous and have been associated with cassava peel degradation (Visagie *et al*., 2014; Ayansina *et al*., 2017).

Submerged fermentation of filamentous fungi is preferred for the production of industrially important enzymes as compared to solid state fermentation because of its inherent advantage of producing higher yield and improved oxygen circulation (Ruban *et al*., 2013; Krishma and Radhathirumalaiarasu, 2017).
Nutrients usually influence the production of primary metabolites especially nitrogen sources. Different nitrogen sources such as Yeast extract, peptone, urea, ammonium sulphate and sodium nitrate have been utilized for amylase yield (Shah *et al*., 2014). Though many studies on amylase production have been done with many fungi including Aspergillus and Rhizopus species (Oshoma and Ikenebomeh, 2005; Oshoma *et al*., 2010, 2017; Abdelwahab, 2015) very few studies have been done with Penicillium species. Hence, effect of different nitrogen sources on the growth and amylase yield of *P. chrysogenum* was investigated using cassava whey.

## Materials and methods

*Sample collection and preparation*: Cassava whey was collected from a local cassava mill in Ugbowo, Benin City, Edo state, Nigeria and was transported to the laboratory for immediate use. The whey was then filtered with cheese cloth, and then sample was diluted with distilled water at a ratio 1:3 (v/v). The diluted sample was then gelatinized with heat. The gelatinized sample was designated as cassava whey medium (CWM).
*Collection and Inoculum preparation of fungus isolate*: The fungus isolate *Penicillium chrysogenum* was collected from the Culture Collection Centre of Microbiology Department, University of Benin, Benin City, and was identified based on cultural and microscopy characterization following standard methods of Barnet and Hunter, (1972) and Larone, (1986). It was then grown on Potato Dextrose Agar (PDA) plates at 28 ± 2 ºC for 3 days, after which the spores were harvested by flooding the plate with 10 mL of sterile 1% v/v tween 80 solution to dislodge the spores from the hyphae. The solution with spores was filtered with a sterile muslin cloth to remove any hyphal fragments present (Oshoma *et al*., 2017). The harvested spores were counted using a Haemocytometer under X40 magnification and inoculum size of $10^6$ spore/mL was used for the fermentation process.
*Media preparation with nitrogen supplementation and fermentation process*: Cassava whey medium (CWM) was used in this study with different trial media. Four trial media were used, each containing Urea (2 g/L), Glycine (1 g/L), $(NH_4)_2SO_4$ (1 g/L) and $NH_4NO_3$ (2 g/L) and made up to 1 L with CWM respectively. The control was made of cassava whey without N supplementation. In all the different media initial pH was adjusted to 4.5 using 1N $H_2SO_4$ and/or 1N NaOH. From the adjusted pH samples 100 ml were transferred into 250 mL Erlenmeyer flasks in duplicates sterilized at 121ºC for 15 min and allowed to cool. After cooling, 500 µl of the inoculum containing $10^6$ spore/mL was aseptically introduced into appropriate flasks. Flasks were then placed on orbital shakers at temperature of 28±2 ºC at 110 rpm. Fermentation was monitored for a period of 8 d, and cell biomass, amylase activity, reducing sugars, pH and titratable acidity were analyzed at regular intervals.
*Analytical methods*: Cell biomass of *Penicillium chrysogenum* was quantified, according to the method described by Oshoma and Ikenebomeh (2005).
The pH values of each of the fermentation media were determined using pH meter (HANNA Instruments) after standardization with buffer at pH 4.
Amylase assay was determined using a modified method of Smith and Roe (1948). Fermentation media were centrifuged at 10,000 rpm for 10 min at 4ºC and supernatants were collected for amylase activity quantification. Phosphate buffer (0.5 M) at pH 8.0 was prepared, and 3 mL of it was mixed with 5.0 mL of 1.2 % starch solution, and 1 mL of 0.5 M NaCl into 2 test tubes labelled A (the digest) and B (the control), with a third test tube labelled C (blank) having same except for 5 mL of distilled water. All test tubes were heated in a water bath at 37 °C for 10 min to equilibrate, and 1.0 mL of enzyme solution (test sample) was then added to tube A to initiate the reaction process. Test tubes were then incubated at room temperature for 30 min, after which 2.0 mL of 0.1M HCl was added across test tubes to terminate reaction process. One milliliter (1 mL) of enzyme solution were added in tubes B (control) and C (blank) and mixed thoroughly. Each of the reaction mixture (2 mL) was transferred into 500 mL volumetric flask containing 400 mL distilled water and 5 mL of normal HCl to which iodine reagent (1 mL) was added and made up to volume. The resulting blue solutions were read at 620 nm using spectrophotometer.
The amylase unit is defined as the amount of enzyme that under the conditions of this procedure, with 60 mg of starch present will hydrolyze 10 mg of starch in 30 min to a stage at which no colour is given with iodine at 620 nm.
Determination of reducing sugars was done using Somogyi-Nelson (1952) method which is based on absorbance at 620nm of a coloured complex between a copper oxidized sugar and arsenomolybdate (Gusakov *et al*., 2011).
The titratable acidity was determined using the standard volumetric titration method. 5 mL of each different media were transferred into 100 ml conical flasks with the addition of two drops of phenolphthalein as indicator.

This was followed by the addition of 0.1 M NaOH which was titrated against each fermentation media until a noticeable color change (pink) is observed, this indicates the end point (Neutralization point).
*Data Analyses*: All experiments were performed in duplicates and values were reported as standard deviations. Differences in media were analyzed using IBM SPSS statistics package 16.0 (SPSS Inc. and IBM Company, Chicago, USA) where $P < 0.05$ implies significant difference between values obtained. All graphs were plotted with sigma plot 10.0.

## Results

Biomass yield of *P. chrysogenum* in different cassava whey media during 8 d fermentation period is presented in Fig. 1. Cell biomass increased with time in the different media. The highest ($P < 0.05$) biomass yield (5.0 g/L) was observed in $NH_4NO_3$ supplemented media as compared to other N amended media and the un-supplemented media (3.0 g/L) at 8 d. Un-supplemented media had significantly lower ($p < 0.05$) biomass yield at 4 and 8 d as compared to the nitrogen supplemented media (Figure 1).

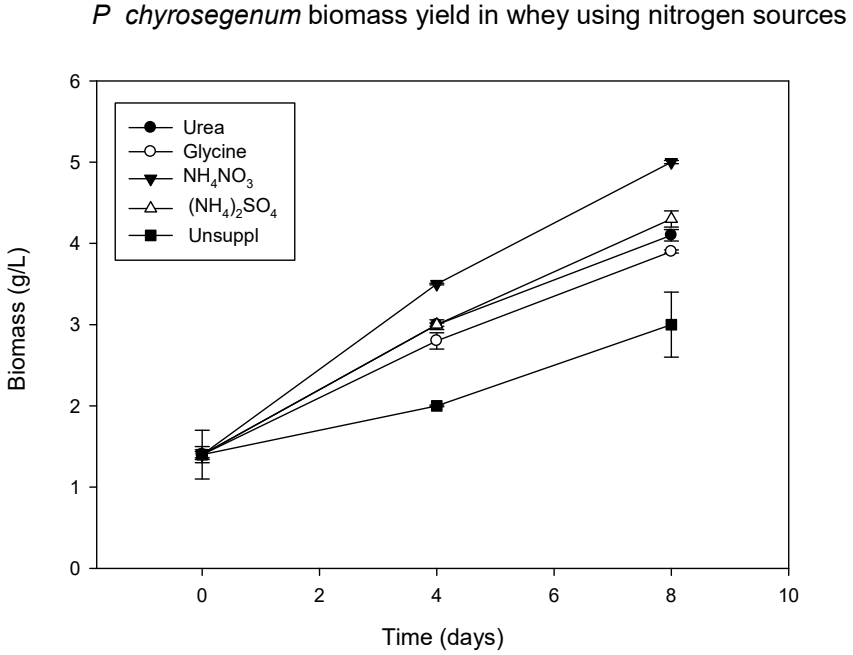


Figure 1: Biomass yield of *P. chrysogenum* in cassava whey media using nitrogen supplementation.

pH values during *P. chrysogenum* growth in the different fermentation media is shown in Table 1. A significant increase ($P < 0.05$) in pH values were observed with time in the different nitrogen supplemented media. However, the unsupplemented media had the highest ($P < 0.05$) pH value at 8 d as compared to the different N amended media (Table 1).

Table 1: pH values during *P. chrysogenum* growth in cassava whey media at different nitrogen supplementation

| Day | Urea | Glycine | $NH_4NO_3$ | $(NH_4)_2SO_4$ | Unsupplemented |
|---|---|---|---|---|---|
| 0 | 4.5 | 4.5 | 4.5 | 4.5 | 4.5 |
| 4 | 5.0 | 4.7 | 4.6 | 4.7 | 4.8 |
| 8 | 5.3 | 4.8 | 4.7 | 4.9 | 6.8 |

Amylase yield increased with time in the different media (Fig, 2). All five media had increased amylase yield at day 8 however, ammonium nitrate ($NH_4NO_3$) supplemented media had the highest yield (8.0±0.04 mU/g) while

the lowest (1.71±0.01 mU/g) was from the un-supplemented medium. Amylase yield from $NH_4NO3$ supplemented medium was higher ($p < 0.05$) than those from other nitrogen supplemented media.

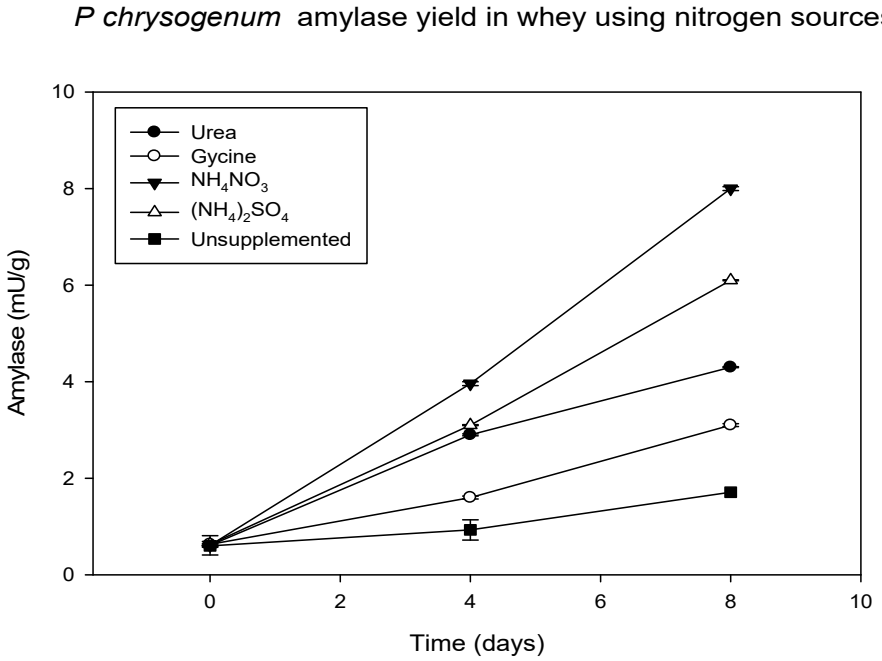


Figure 2: *P. chrysogenum* amylase yield in cassava whey using nitrogen supplementation.

Reducing sugar decreased in all fermentation media at 8 d as compared to 0 d but was significantly lower ($P < 0.05$) in $NH_4NO_3$ supplemented media compared to the organic N supplemented and un-supplemented media.

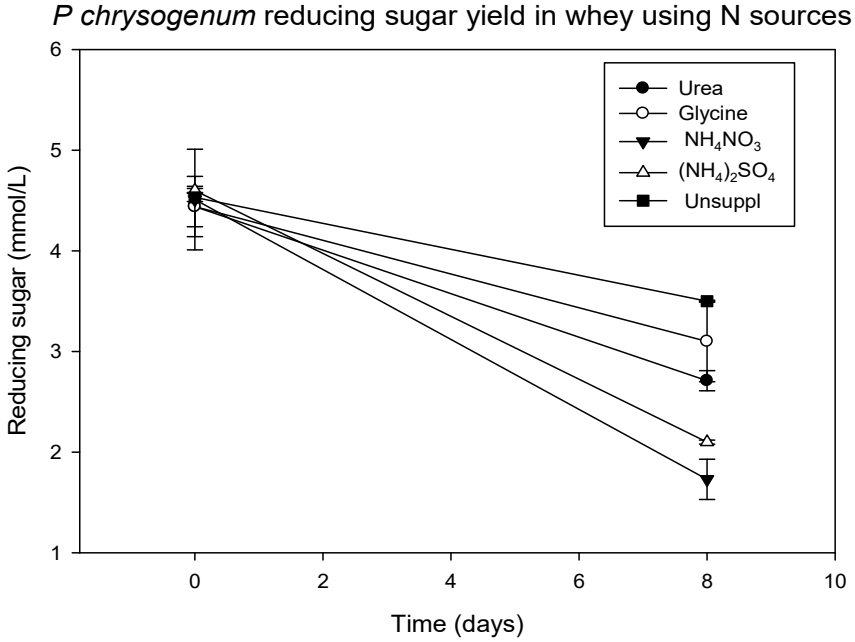


Figure 1: *P. chrysogenum* reducing sugar yield in cassava whey using nitrogen supplementation.

Titratable acidity (TA) during *P. chrysogenum* growth in fermentation media is shown in Table 2. Percentage titratable acidity decreased with time, with urea supplemented media having the lowest titratable acidity (0.11 %) at 8 d. Un-supplemented media had constant TA during the fermentation period but was least ($P<0.05$; 0.11 %) on day 8 compared to other media.

Table 2: Titratable acidity (%) during P. chrysogenum growth in cassava whey at different nitrogen supplementation

| Day | Urea | Glycine | $NH_4NO_3$ | $(NH_4)_2SO_4$ | Unsupplemented |
|---|---|---|---|---|---|
| 0 | 0.27 | 0.31 | 0.33 | 0.29 | 0.33 |
| 4 | 0.23 | 0.27 | 0.23 | 0.25 | 0.33 |
| 8 | 0.21 | 0.23 | 0.21 | 0.17 | 0.11 |

## Discussion

*P. chrysogenum* growth and amylase yield was optimized in cassava whey media using two organic (urea and glycine) and inorganic ($NH_4NO_3$ and $[(NH_4)_2SO_4]$) nitrogen sources. Fermentation media were prepared and tested for biomass and amylase yield, with reducing sugar, titratable acidity and pH over 8 day-submerged fermentation period.

Cell biomass increased with time in the different media because cassava whey is a good source of carbon and therefore, provides suitable conditions for the growth of microorganisms (Krishma and Radhathirumalaiarasu, 2017). Reduced cell biomass could be experienced with time, however, as a result of depletion of nutrients in fermentation media or substrate inhibition (Ogbonna *et al*., 2015; Ayansina *et al*., 2017) The higher biomass yield observed in N supplemented media at 8 days compared to the unsupplemented media could be because nitrogen supplementation helps to augment fungal metabolism and cell growth as well as amylase yield (Padmavathi *et al*., 2012; Shah *et al*., 2014; Oshoma *et al*., 2010). Since cassava whey is poor in protein content addition of nitrogen supplements would be expected to enhance biomass yield (Ugwu and Odo, 2008; Oshoma *et al*., 2010). The low biomass yield in un-supplemented media shows the importance of supplementation in increasing biomass yield and could be as a result of limited concentration of nutrients especially N which is required for growth and biomass formation (Oshoma *et al*., 2005)

Higher biomass was observed with $NH_4NO_3$ supplemented medium compared to organic N sources. This finding agrees with that of Oshoma *et al*. (2017) where $NH_4NO_3$ was reported to crop the highest biomass of *A. niger* compared to other N sources. Anupama and Ravindra (2001) reported that $NaNO_3$, was an effective nitrogen supplement source during the production of single cell protein by *A. niger* using solid state fermentation technique. However, this was in contrast with the report of Ogbonna *et al*. (2015) where it was stated that organic nitrogen (peptone) supplemented medium produced the highest cell biomass. Oshoma *et al*. (2010) similarly reported highest biomass yield of *A. niger* in yeast extract compared to $NaNO_3$ and $(NH_4)_2SO_4$.

Amylase yield increased with time in the different media with $NH_4NO_3$ supplemented medium having the highest amylase yield at 8 days compared to organic N sources and the un-supplemented media. This could be because ammonium salts are known stimulators of microbial growth and amylase production, nitrogenous compounds are also essential macronutrients for nucleic acids and protein biosynthesis (Oshoma *et al*., 2017). Oshoma *et al*. (2017) had similar observations with $NH_4NO_3$ amended fruit waste media which had higher amylase yield compared to other inorganic N sources. Shah *et al*. (2014) and Oshoma *et al*. (2005) however, reported yeast extract and ammonium sulphate as best N sources for amylase production respectively. Ammonium sulphate $[(NH_4)_2SO_4]$ enhanced amylase production by Rhizopus sp in rice bran fermentation media (Akpan *et al*., 1996). Several authors have also reported that organic nitrogen sources were best for amylase production (Erdal and Taskin, 2010; Vidya *et al*. 2012). $NH_4NO_3$ supplemented medium had the highest amylase yield at 8 days of fermentation. Different investigators have reported maximum amylase yield at different days using fungi; for example, Ellaiah *et al*. (2002) and Ruban *et al*. (2013) reported maximum amylase production at day 5 while Singh *et al*. (2009) and Vidya *et al*. (2012) reported maximum amylase production at day 6. These differences could be as a result of different growth rate of the fungi as well as the starting inoculum load.

Cassava is a highly starchy food and therefore had high reducing sugar values at unfermented state. However, the reduction at 8 days of fermentation could be because of *P. chrysogenum* growth and metabolism. Qureshi *et al*. (2004) reported a decrease in reducing sugar concentration with increase in growth of *P. lilacinum* in mineral medium with sugar. Sugar utilization in the media by *P. chrysogenum* increased the production of amylase.

The production of amylases by microbes is affected considerably by physical and chemical parameters of the medium such as pH, and optimum pH is a critical factor for the stability of enzyme produced (Ruban *et al*., 2013; Ajita and Thirupathihalli, 2014; Oshoma *et al*., 2010; Roohi- Kuddus, 2014; Krishma and Radhathirumalaiarasu, 2017). Generally, pH values increased with time in the fermentation media with $NH_4NO_3$ and $(NH_4)_2SO_4$ supplemented media having pH values of 4.7 and 4.9 respectively at 8 days of fermentation while un-supplemented media had pH increase of 2.3 unit (Table 1). Percentage titratable acidity had a corresponding decrease of 0.21 % and 0.11 % in $NH_4NO_3$ and unsupplemented media respectively from 0.33 % at 0 d. The increase in pH values with time is in agreement with the findings of Oshoma *et al*. (2017) where increase in pH values with time was reported during fermentation of different fruit wastes in different nitrogen sources with *A. niger*. pH values are also close to that reported by Oshoma *et al*. (2010) in yeast extract and $(NH_4)_2SO_4$ supplemented cassava whey media using *A. niger*. Generally, fungi prefer slightly acidic conditions because of its influence in the solubility of medium substrates. The results showed an acidic pH values for all the different N supplemented media. Amylases are generally stable over a wide range of pH from 4 - 11 (Vidya *et al*., 2012; Gowhar *et al*., 2015; Ayansina *et al*., 2017). Ayansina *et al*. (2017) reported optimum amylase activity at pH 6 for Aspergillus sp while Ruban *et al*. (2013) reported maximum amylase yield of 8.91 mg/ml/min from sago waste at pH 5 using *A. niger*. The optimum pH value for NH4NO3 supplemented media which had the highest amylase activity was 4.7. Nitrogenous sources have been reported to increase acid production in the medium and might eliminate the need for pH control thereby, ensuring less competition for the available nutrients in the substrate and promoting higher biomass (Ikenebomeh and Chikwendu, 1997; Oshoma *et al*., 2005; Oshoma *et al*., 2010).

## Conclusion

The present study showed that *P. chrysogenum* is a good source of amylase under submerged fermentation process (SmF) using cheap agricultural waste. Amylase activity was optimized with $NH_4NO_3$, and this highlights the importance of nitrogen supplementation during amylase production especially when using cheap carbon sources that have limited N content. Use of cassava whey and *P. chrysogenum* is a cost effective way of producing amylase and an eco-friendly way of waste disposal.

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