

Production of amylase by *A. niger* under submerged fermentation using pineapple peel powder as the substrate and its application in baking industry

H. M. J. Pushphakumara, M. K. B. Weerasooriya *

Faculty of Science, University of Kelaniya, Sri Lanka, *bandu@kln.ac.lk

Abstract

Starch-degrading amylase was produced by *A. niger* under submerged condition utilizing dried pine apple peel powder as the substrate. Growth parameters were optimized by changing the incubation period, pH of the culture media, level of carbon source (dried pineapple peel powder) and additional nitrogen source, in order to get a maximum amylase production. The study revealed that the production of extracellular amylase increased with the culture growth and reached to the maximum level at day 7 in the cultures grown at pH 5.0. Studies with different levels of pine apple peel powder ranging from 6g/L to 48g/L indicated that the optimum level of carbon source for the maximum amylase production was 18 gL⁻¹. The effect of additional nitrogen source on amylase production was also monitored supplementing the growth media with different nitrogen sources such as peptone, gelatin and urea. The result indicates that using gelatin as additional nitrogen source increases the amylase production. Experiments were also carried out to monitor extracellular and intracellular amylase production and the results revealed that the extracellular amylase production was found to be higher than that of intracellular. The yield of the enzyme was 8530 units/g of pine apple peel powder.

To test the suitability of the enzyme for the improvement of the quality of bakery products were studied by treating the wheat flour dough with different volumes of partially purified enzyme. The results showed that the treating 7.7 mL (11.5 U mL⁻¹) partially purified amylase with one kg of wheat flour dough provides better quality product with improved shiny appearance, color, crumb structure, taste and better anti-staling effect than the control.

Keywords; *amylase, pine apple peel, submerged fermentation, bakery products, antistaling effect*

Introduction

Amylase stand out as a class of enzyme that useful in food, brewing, textile, detergent and pharmaceutical industries. Though amylase can be derived from various sources such as plants, animals and microorganisms, enzymes from microbial sources have been dominated in industrial sectors because of their economical bulk production capacity and the ease of manipulation. *Aspergillus niger* is one of the organisms widely used for the commercial production of amylase, (Suganthi *et al*, 2011).

Fungal amylases particularly from *Aspergillus species* find various application in food industry; eg.as an anti-staling agent in baking industry, for haze clarification in fruit juices, alcoholic beverages and maltose syrup production etc. Several methods such as submerged fermentation and solid-state fermentation have been successfully used for the amylase production from various micro-organisms .Since, the contents of synthetic medium used for amylase production are very expensive and uneconomical, they need to be replaced with more economically available agricultural and industrial by products. Agro industrial residues such as wheat bran, spent brewing grain, maize bran, rice bran, rice husk, coconut oil cake, mustard oil cake, corn bran *etc.* have been used as substrates for the amylase production (Krishna *et al.* 2012; Suganthi *et al.*, 2011;).

Thus, the present study was designed to produce amylase by *A. niger* under submerged fermentation using pine apple peel powder as the carbon source. In this work, amylase production will be optimized by changing the parameters such as the incubation time, pH, the level of carbon source and the nitrogen source. Amylase produced under optimized conditions will be partially purified and its potential to improve the quality of bakery products will be investigated.

Methodology

Culture conditions and growth

Aspergillus niger strain was obtained from the Department of Microbiology, University of Kelaniya

Fungal culture was maintained on stock slopes. Fresh Pineapple peel (250g) were cleaned, well dried and powdered.

Preparation of sub-culture media

Glucose 20g, Yeast extract 1.0g, NH_4Cl 2.5g, KH_2PO_4 0.3g, NaCl 0.25g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1g prepared in 1L (distilled water). pH was adjusted to 5.6 (Rezaei *et al.* 2009) autoclaved at 121 °C for 15 min. Ten ml of sterile distilled water added to one stock slopes shake well inoculated the sterile culture flask which contained 25ml of above media. Thus inoculated flask was incubated and was shaken at 200rpm for 24 hrs.

Amylase production with the culture growth at different pH level

Culture media 1L was prepared using the above methodology. Dried pine apple peel powder was used instead of glucose. pH was adjusted to 5.6, autoclaved at 121 °C for 15 minutes, inoculated with 2ml of subculture and incubated for 10days. Two culture flasks were taken at day 4, mycelia was filtered and the filtrate was assayed for amylase activity. Likewise, two flasks were taken at 5th, 6th, 7th, 8th, 9th, 10th days of inoculation and mycelium was filtered. Filtrate was assayed for amylase activity. Control experiments were also carried out. All the assays were done in triplicate. Mean and the standard deviation were calculated. All the steps in above procedure were repeated under the same conditions changing only the pH of culture media. Similar experiments were performed at pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 also.

Amylase production with different levels of pineapple peel powder

Culture flasks which contained above culture medium (pH 5.0) were prepared supplementing with different concentrations of Carbon source (6 gL⁻¹, 12 gL⁻¹, 18 gL⁻¹, 24 gL⁻¹, 30 gL⁻¹, 36 gL⁻¹ and 42 gL⁻¹), autoclaved at 121 °C, 15 lb/inch² for 15 min. Each flask was inoculated with sub culture (1.00 mL) and incubated at room temperature for 10 days. Amylase production at each substrate level was monitored from day 4 to 10 as in above procedure.

Amylase production with additional Nitrogen (N) source

Fungus was grown in 12 culture flasks which contained the above medium (pH 5.0) supplemented with pineapple peel powder 18 g/L. Three flasks were used as a control and other nine flasks were supplemented with additional N sources eg. peptone, urea and gelatine. Amylase production was monitored at day 7. All the replicates were contained three samples and assays were done in triplicate. Mean and the standard deviation were calculated.

Comparison of extracellular and intracellular amylase activity of the fungus

A.niger was grown in 14 culture flasks (250mL) in which each contained 50mL culture media and all the flasks were incubated for 10 days under optimized conditions to produce amylase enzyme.

After 4 days of inoculation, 2 conical flasks were taken, and the mycelia were filtered. Amylase activity of the culture filtrates was assayed. That was considered as extracellular enzyme activity. Fungi mycelium was used to extract for intracellular amylase. For this, fungi mycelium was ground with little amount of cleaned sand and sodium phosphate buffer (0.02M, pH 6.5). The mixture was ground well until it becomes slurry. Buffer (10.0 mL) was added to the slurry and it was centrifuged at 5000 g for 15 min. Amylase activity of the supernatant was assayed. That was considered as intracellular enzyme activity.

Another 2 flasks were taken at 5th day similarly and the mycelium was filtered, and amylase activity of the culture filtrates were assayed for the extracellular enzyme activity.

Intracellular enzyme was extracted from fungi mycelium and was assayed for amylase activity. Likewise, after 6th, 7th, 8th, 9th and 10th days of inoculation two flasks were taken on each day, extracellular and the intracellular amylase activities were detected.

Amylase assay

Culture broth 30 µL and 0.2 mL of 0.1% starch solution were mixed. Then volume was adjusted up to 0.3 mL by using 70 µL of distilled water and above mixture was incubated at 30 °C for 15 min. After 15 min incubation time, 0.5 M acetic acid 1.00 mL was added to stop the reaction. Amylase activity was determined by adding to the, 0.3 mM Iodine/KI reagent 0.7 mL, mixed well and the absorbance was measured at 620 nm. Amylase enzyme activity (U/mL) was defined as (Maryam *et al.* 2008),

$$U/mL = \frac{(\text{Absorbance}_{620\text{ nm}} \text{ of control} - \text{absorbance}_{620\text{ nm}} \text{ of sample})}{(\text{Absorbance}_{620\text{ nm}} 1 \text{ mg starch} \times t \times v)}$$

t = Assay incubation time v = Added enzyme volume

Industrial application of bread making

Wheat flour 2000g, Salt 40g, Yeast 50g, Sugar 40g and Water 1200 mL

All ingredients were mixed by using spiral mixture for 4 min to prepare the dough. Thus prepared dough was divided into 450 g portions. Different volumes of enzyme 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 mL were added into each dough sample and mixed well. One sample was left as the control. Then well mixed dough samples left for 20 min at room temperature 28 °C. Each dough sample was divided into 3 equal weighted portions and each portion was placed in metallic plate without cover and incubated at room temperature for 2 hours. Then all the portions were baked at 220 °C for 25 minutes in an oven. Then dough colour, appearance, taste of enzyme added baked sample and staling effect were observed and compared with control sample.

Results and Discussion

The optimization of various parameters and the manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet the industrial demands. Various physical and chemical factors have been known to affect the production of α -amylase such as the temperature, pH, period of incubation, the carbon sources and the nitrogen sources. Hence, the production of the enzyme under the above parameters was investigated.

Kinetics of enzyme production with the culture growth at different pH levels

Figure 1.a shows the variation of amylase enzyme activity with culture growth at different pH levels at 28 °C.

Enzyme production was increased with culture growth and reached to a maximum at 7th day (figure 1.a). Similar result has been reported for *A.niger* grown in Banana peel (Rajilla *et al.* 2013). Gupta *et al.* (2008) reported different results in amylase production under pH 5. In their study *A.niger* grown on starch at 30 °C showed maximum enzyme production at 5th day. As reported by Adejuwon. (2010) *A. niger* grown on Citrus fruit isolate showed maximum amylase production after 10 days of incubation at 35 °C.

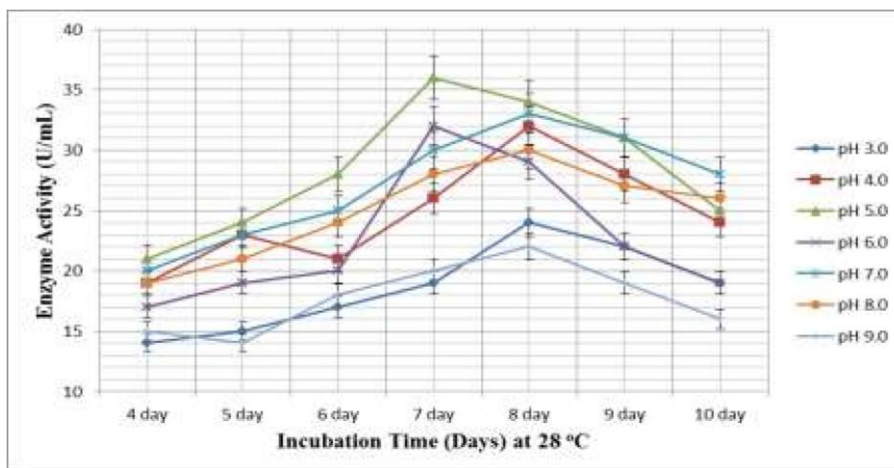


Figure 1. a) Kinetics of amylase production at different pH levels at 28 °C

These finding indicates that the amylase production is active in the pH range of 4.0- 8.0, suggesting that the enzyme would be useful in processes that required wide range of pH change from slightly acidic (pH 4) to slightly alkaline (pH 8) range. Similar results have been observed in the crude amylase preparation in Bergmann *et al.*, 1998; Hayashida & Teramoto. 1998.

Using the data obtained in above experimental amylase production in 7 days old cultures at different pH level are summarized in figure 1.b

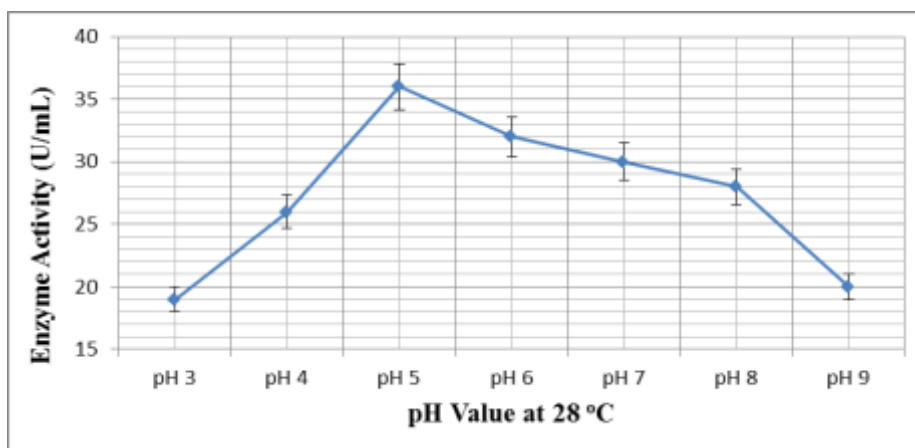


Figure 1.b). Effect of pH level on enzyme production at 7th day in 28 °C

Similar result has been reported with *A.niger* grown in Banana peel, corn, potato and tapioca at 30 °C (Darani& Kumaran, 2012). Gupta *et al.* (Gupta *et al.* ,2008) also describe similar result for *A.niger* grown in starch at 30 °C.

However, *A.niger* grown on *Ipomoea batatas* at 28 °C showed different result. In that work pH 7 has been shown maximum enzyme production (Sundar *et al.* 2012). Another different result has been reported with *A.niger* grown on citrus fruit isolate at 35°C. In that study pH 6.5 has been given the optimum amylase activity (Adejuwon, 2010).

Alpha amylases are generally stable over a wide range of pH from 4 to 11 (Fogarty & Kelley, 1980) and most *Bacillus* isolates, e.g. *B. subtilis*, *B.licheniformis* and *B.amyloliquefaciens* seem to require an initial pH of 7.0 (Haq *et al.* 2002).

Effect of substrate level for maximum enzyme production

Studies were done with different substrate levels in order to get the maximum yield of amylase enzyme (Fig.3).

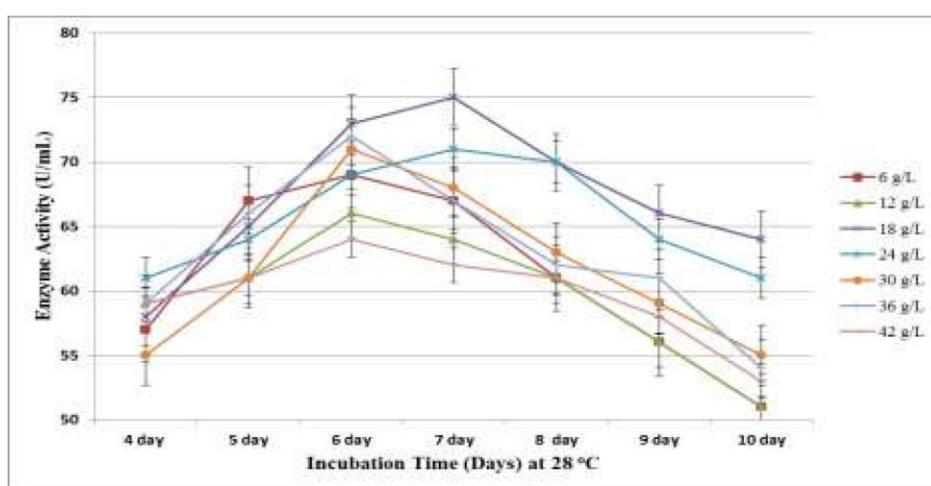


Figure 3. Effect of substrate level for maximum enzyme production at 28 °C

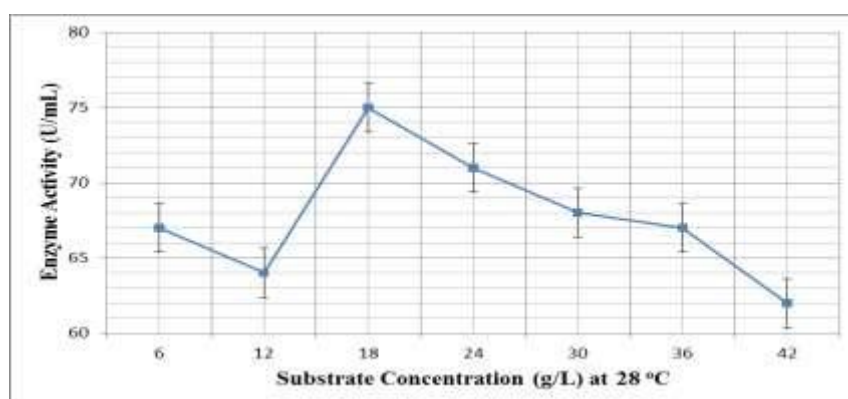


Figure 4: Effect of substrate concentration on the production of amylase at 7th day in 28 °C

As shown in figure 4 enzyme productions increased with substrate level and reached to maximum at 18 g/L level. Above that level enzyme production was gradually decreased.

As reported in Krishna *et al*, *A.niger* grown on Banana peel at 28 °C for 7 days have been shown maximum enzyme production at 25-30% concentration of Banana peel (Krishna *et al.* 2012).

However *Aspergillus sp.* JGI 12 grown on using 1% starch in Coconut oil cake, Ground nut oil cake and Rice bran as the substrate at 25 °C for 6 days showed different result. Rice Bran gave the highest enzyme activity (Glucoamylase 16.42 U/mg) (Alva *et al.* 2007).

Effect of additional Nitrogen (N) source for enzyme production

Supplementing the culture broth with additional nitrogen source increased the amylase production as shown in figure 3.5. Similar result reported in Suganthi& Coworkers. In their work additional nitrogen source has increased the yield of alpha amylase produced in ground nut oil cake medium with *A.niger* grown at 37 °C (Suganthi *et al.* 2011).

In the present study, culture broth supplemented with gelatin (20 g/L) showed maximum amylase production compared to other nitrogen sources. Culture broth supplemented with peptone showed similar results in comparison with control experiment (culture broth with no additional nitrogen source) indicating that peptone has no stimulating effects on amylase production.

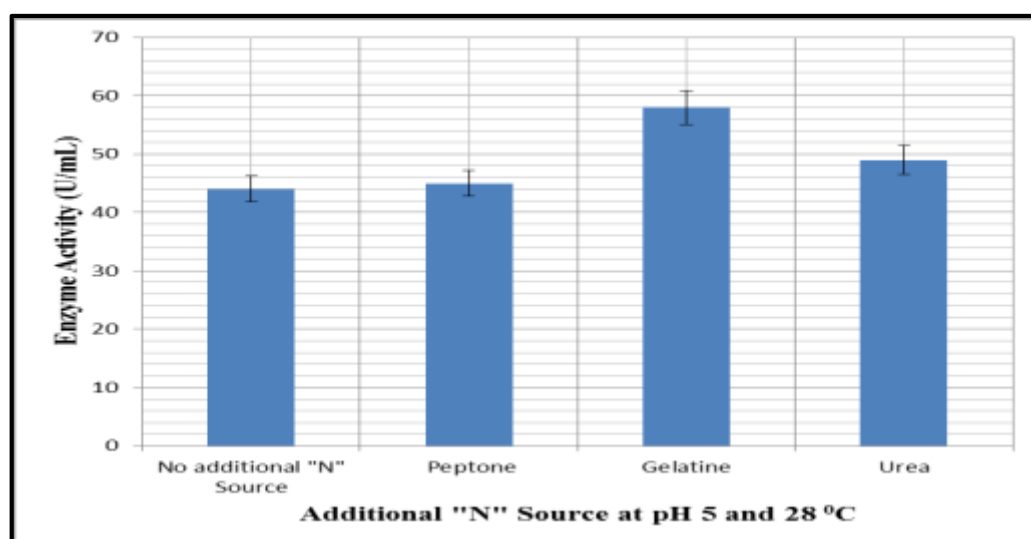


Figure 5: Effect of additional N source for enzyme production at pH 5 and 28 °C

This finding suggests gelatin is suitable as an additional nitrogen source for the production of amylase by *A.niger* grown on Pineapple peel.

In earlier study, the effects of additional nitrogen sources on the production of α -amylase were observed by Gupta & his coworkers (2008).

In their study casein and gelatin caused poor enzyme production with *A.niger* grown on starch at 30 °C. But as reported in Gupta *et al.* peptone has been supported to enhance amylase production whereas urea also has shown considerable increase of α -amylase production (Gupta *et al.* 2008).

As reported by Varalakshmi *et al.* (2008) *A. niger* grown on wheat bran supplemented with urea has given the maximum amylase activity (61.33U), followed by beef extract(41.33U) and casein (44.35U)but wheat bran supplemented with meat extract resulted in a decrease of enzyme production (Varalakshmi *et al.* 2008).

As reported by Sundar *et al* (2012), *A.niger* grown on *Ipomoea batatas* has been resulted in a considerable increase in the production of alpha amylase upon addition nitrogen sources such as peptone, casein and yeast extract. Out of these nitrogen sources, peptone showed maximum amylase activity compared to casein and yeast extract (Sundar *et al.* 2012).

Determination of the extracellular and intracellular enzyme activity

As presented in fig.6. extracellular and intracellular enzyme production has been reached to maximum at 7th and 6th day respectively. Extracellular enzyme production is found to be always higher than the intracellular production. Most probably this is due to the extra cellular digestion of fungi digest the food first and then ingests the food, to accomplish this by producing exo-enzymes.

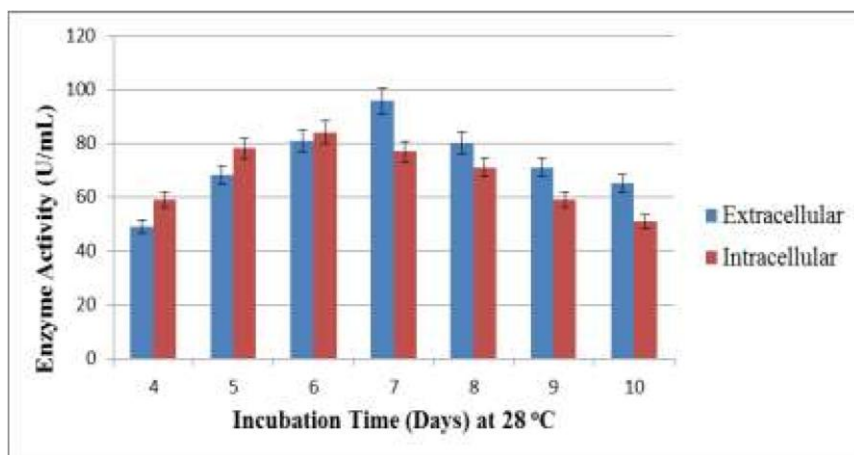


Figure 6: Determination of the extracellular and intracellular enzyme activity at 28 °C

Suitable enzyme volume for baking application



Bread –made from 450g of wheat flour Bread –made from 450g of wheat flour
dough supplemented with 3.50 mL (11.5 u/ml) of amylase dough supplemented with 4.00 mL (11.5 u/ml) of amylase

Figure 7 Appearance of bread with different volume of amylase

Table 1: Bread samples with different volume of enzyme

Property tested	Volume of amylase / mL(amylaseactivity11.5U/ ml						
	0.00	2.00	2.50	3.00	3.50	4.00	4.50
Shiny appearance	0	1	1	2	3	4	3
Crust colour	0	1	2	3	4	4	4
Taste	0	1	2	3	3	4	3

1 = No difference with control

2 = Little better than Control

3 = Better than Control

4 = Much better than Control

Result in table 1 indicate 3.5ml / 450g wheat flour dough is the best treatment to improve the appearance, crust color and the taste of bread.

Table 2 Staling effect in bread

Treatment (T)	Enzyme Volume (ml)	Staling Effect				
		Day 1	Day 2	Day 3	Day 4	Day 5
T _{control}	0.0	No	Start	Yes	Yes	Yes
T ₁	2.5	No	No	Start	Yes	Yes
T ₂	3.0	No	No	Start	Yes	Yes
T ₃	3.5	No	No	Start	Yes	Yes
T ₄	4.0	No	No	No	Start	Yes
T ₅	4.5	No	No	No	Start	Yes

Results given in Table 2, staling effect started in control (T_{control}), at day 1 But staling was commenced after three days in T₁, T₂ and T₃ and after four days in T₄ and T₅. Hence, it could be recommended T₄ (7.7ml/kg, amylase activity 11.5U/ml) is the best volume for retarding the anti staling.

Enzyme yield was 8530units/g pine apple peel powder.

Conclusion

The present experiment revealed the potential of amylase production by *A.niger* under the submerged fermentation using Pineapple peel waste as the carbon source.

The maximum amylase enzyme activity was obtained, seven days after incubation at pH 5 with 18 gL⁻¹ concentration of carbon source. Out of the tested additional nitrogen sources (Gelatin, Peptone and Urea) Gelatin provided the maximum enzyme yield.

Fungal strain produced amylase as an extracellular amylase as well as an intracellular amylase. Extracellular amylase production was found higher in comparison with intracellular amylase production after 7th days incubation period.

Treating 8.8mL(153.24 U/mL) of produced amylase with 1kg of wheat flour dough provided better quality product [4 = much better than control (sensory evaluation mark)] with improved shiny appearance taste and higher anti-staling effect.

Finally findings of this work reveal the potential of using agricultural waste of Pine apple peel in large scale production of industrial amylase. Utilization of these agricultural byproducts will help to solve the pollution problems due to their continuous accumulation and also contribute to safe and economical waste management.

References

- Adejuwon, A. O. (2010). Synthetic production of amylase from *Aspergillus niger* isolated from citrus fruits. Leadcity university, Ibadan, Nigeria. *African journal of basic & applied sci.* 2(5-6): 158-160.
- Alva, S., Anupama, J., Savla, J., Chiu, Y. Y., Vyshali, P., Shruti, M., Yogeetha, B. S., Bhavya, D., Purvi, J., Ruchi, K., Kumudini, B. S. and Varalakshmi, K. N. (2007). Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI12 in solid state culture. Department of Biotechnology, Bangalore. 6 (5): 576-581.
- Bergmann, F. W., Abe, J. and Hizukuri, S. (1998). Selection of micro-organisms which produce raw starch degrading amylases. *Appl. Microbiol. Biotechnol.* 287: 443-446
- Dharani, G., and Kumaran, N. S. (2012). Amylase production from solid state fermentation and submerged liquid fermentation by *Aspergillus niger*. Bangladesh. 47(1): 99-104..
- Fogarty, W. M., and Kelley, C. T. (1980). Amylase, amyloglucosidase and related glucanases in; microbial enzymes and bioconversions. Academic Press, London. pp. 115-170.
- Gupta, A., Gupta, V. K., Modi, D. R., and Yadava, L. P. (2008). Production and characterization of α -amylase from *Aspergillus niger*. Asian network for scientific information. India. 7(3): 551-556.
- Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., and Chauhan, B. (2003). Microbial amylases: a biotechnological perspective. *Process biochem.* 38: 1599-1616.
- Haq, I., Ashraf, H., Abdullah, R., and Shah, A. H. (2002). Isolation and screening of fungi for the biosynthesis of alpha amylase. *Biotechnology.* 2: 61-66.
- Hayashida, S., Teamoto, Y. and Inove, T. (1998). Production and characteristics of raw potato starch digesting amylase from *Bacillus subtilis*. 65.*Appl. Environ. Microbiol.* 54: 1516-1522.

Krishna, P. R., Srivastava, A. K., Ramaswamy, N. K., Suprasanna, P., and Souza, S. F. D. (2012). Banana peel as substrate for α -amylase production using *Aspergillus niger* NCIM 616 and process optimization. *Indian journal of biotechnology*. 11: 314-319.

Maryam Yaldagard, M., Seyed Ali Mortazavi, S. A and FaridehTabatabaie, F. (2008). Effect of ultrasonic power on the activity of Barley's alpha-amylase from post-sowing treat of seeds. Department of Chemical Engineering, Ferdowsi University, Mashhad. *World Applied Sciences Journal* 3 (1): 91-95.

Rajila, C., Liji, T., Sundar, R., and Suganyadevi, P. (2013). Bioprocessing of *Curcuma angustifolia* for α -amylase production by *Aspergillus niger*. Department of Biotechnology, Coimbatore, Tamilnadu, India. 5: 748-752.

Rezaei, P. S., Najafpour, G. D., Shafaghat, H., and Mahjoub, S. (2009). Production of α -amylase from starch using *Aspergillus niger*. *World applied sciences journal*. 7(3): 306-311.

Suganthi, R., Benazir, J. F., Santhi, R., Ramesh, K. V., Anjana, H., Nitya, M., Nidhiya, K. A., Kavitha, G., and Lakshmi, R. (2011). Amylase production by *Aspergillus niger* under solid state fermentation using agro-industrial wastes. *International Journal of Engineering Science and Technology (IJEST)*. 3: 1756-1763.

Sundar, R., Liji, T., Rajila, C. and Suganyadevi, P. (2012). Amylase production by *Aspergillus niger* under submerged fermentation using *Ipomoea batatas*. Tamilnadu, India. 3: 175-182.

Varalakshmi, K. N., Kumudini, B. S., Nandini, B. N., Solomon, J. D., Mahesh, B., Suhas, R., and Kavitha, A. P. (2008). Characterization of Alpha Amylase from *Bacillus* sp. isolated from paddy seeds. *Journal of applied biosciences*. 1(2): 46-53.