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SELECTION OF ACID AND BASE TREATMENT OF SUGARCANE WASTE AS CARBOON SOURCE FOR THE PRODUCTION OF AMYLASE BY *PENICILLIUM EXPANSUM*

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ABSTRACT: **S**ugarcane wastes were (bagasse and Leaves) pretreated with 0.6N acid and base and the hydrolysates were utilized as carbon source for the growth of *Penicillium expansum* and the production of amylase. It was found that maximum yield of amylase was achieved (2.6208 units/ml) at 168 hours when Penicillium expansum was grown on pretreated KOH sugarcane leaves mineral medium.

INTRODUCTION: Starch is composed of a mixture of linear and branched poly-saccharides consisting of α-1, 4-glucosyl residues and α-1, 6 – glucosyl branch point [1]. The glucose production from starch involves a number of amylolytic enzymes in a two-step process (α-amylases, α-gluco-sidase, pullulanases) while each has a different pattern of action on starch. In the first step (liquefaction), concentrated starch suspension (30-40%) is converted into solution of soluble dextrins having different degree of polymerization. In the second step (saccharification), these dextrins are hydrolyzed to glucose as shown in Figure - 1 [2]. Amylases are multi-enzymic system, which contain α-amylase (endo-amylase), β-amylase (exo-amylase), γ-amylase (amylo-glucosidase) and debranching enzymes such as Pullulanase and Isoamylase [3,4]. The α-amylase or endo-amylase hydrolyses α-1, 4-glucosidic bond in amylose, amylose pectin to mixture of glucose (e.g. maltose, oligosaccharides

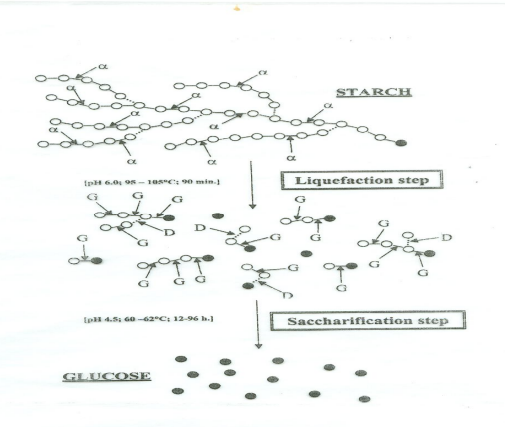


Figure 1. Mode of action of amylolytic enzymes on starch

and Dextrin), which rapidly reduces the viscosity of gelatinized starch (Lique-faction) but doesn't attack 1,6-glucosidic linkages in branch polymer of starch.

The β-amylase or exo-amylase hydrolyze α-1,4-glucosidic bond, but cannot by pass α- 1,6 linkages of starch, amylose and amylopectin and this enzyme

cleaves alternate glucosidic bond starting at the non-reducing end and producing maltose.

The γ-Amylase or Amylo-glucosidase produces β-D-glucose from non reducing chain ends of amylose, Amylopectin and glycogen by hydrolyzing α-1,6 and α-1,3 linkages at the slow rate and it is possible to achieve 100% conversion of starch to glucose using combination of α-amylase and glucoamylase (amylo glucosidase).

The debranching enzymes are capable of degrading amylopectin and other polymers containing α-1,6 glucosidic linkages. There are two types of debranching enzymes. Pullulanase can hydrolyze pullulan, amylopectin and dextrin but cannot degrade native glycogen while Isoamylases are capable of hydrolyzing debranching glycogen, but can not degrade pullulan (pullulan is a polymer of α-1,4 and α-1,6-linked glucose in which the α-1,6-bond occurs more or less after every third glucose unit.

The main object of this study is to utilize sugar cane waste, which does not have any beneficial use. In present study, work is under taken to find out effective acid or base treatment method for sugar cane waste and use hydrolysate as a carbon source for the production of amylase by Penicillium expansum.

MATERIAL AND METHODS:

Microorganism: *Penicillium expansum* was isolated and identified in Fermentation Biotechnology Research Laboratory, Department of Chemistry Laboratory, Shah Abdul Latif University, Khairpur Mirs, Pakistan. The stock culture was maintained on Czepak's agar. The sterilized slants were inoculated with *Penicillium expansum* and incubated at 27ºC to obtain luxuriant growth.

Chemicals: Cellobiose, sodium potassium tartrate and DNS were purchased form BDH, E. Merck and Sigma chemicals respectively. All other reagents used were of analytical grade.

Culture medium: The following ingredients were used for the preparation of culture medium as reported by Burrel et al [5] without changing the chemical composition using g/L of $(NH_4)_2SO_4$ 2.5g; fumaric acid 2.0g; KH_2PO_4 1.0g; $MgSO_4.7H_2O$, 0.5g; (NH_4) Fe $(SO_4)_2.12H_2O$, 0.2mg; $ZnSO_4.7H_2O$, 0.2mg; $MnSO_4.5H_2O$, 0.1mg and thiamine hydrochloride 0.1mg. The pH of the culture medium was adjusted to 6.0.

Preparation of spore suspension: To stock culture of *Penicillium expansum*, 10ml of sterilized water was added and the surface was gently rubbed with sterilized wire loope. The spore suspension was further diluted to 100ml with sterilized water [6].

Hydrolysis of sugarcane waste: 10g of sugarcane waste such as bagasse and sugarcane leaves were hydrolyzed with 800ml of 0.6N H_2SO_4, HCl, HNO_3, $HClO_4$, NaOH and KOH for two hours on flame, maintaining the level of slurry constant. The digested slurry was autoclaved for 30 minutes at 15 kg/ Cm^2.The slurry was filtered through Whatman No. 1 filter paper after cooling at room temperature. The filtrate of solubilized sugarcane waste was incorporated with mineral medium as a carbon source. The loss in weight of sugarcane waste was determined after drying at 105°C to constant weight.

Cultivation condition: 50ml of solubilized sugarcane waste incorporated with culture medium was taken in 250ml conical flasks plugged with cotton wool and autoclaved at 1.5 kg/Cm^2 for 20 minutes. The sterilized media cooled at room

temperature was inoculated with 1ml of *Penicillium expansum* spores. The flasks were incubated in an orbital cooled shaking incubator at 28 ± 2°C adjusted at 200 rev min^{-1}. The culture broth was separated from mycelium after an interval of 24 hours incubation period by filtration through Whatman No.1 filter paper. The enzyme activity of amylase was examined in the culture broth. The mycelium was dried at 105°C in an oven to constant weight.

Determination of total carbohydrate: The carbohydrate content of digested sugarcane wastes and culture broth was measured by phenol sulphuric acid method [7] with glucose as the standard.

Assay of α-amylase activity: Amylase activity was determined by the method of Bernfeld *et al.*, [8]. 1ml of enzyme sample (culture broth) was mixed with 1ml of 1% soluble starch and 2ml of sodium acetate buffer pH 4.6. The reaction was carried out at 35°C for one hour. The reducing sugars produced were estimated by dinitrosalicylic acid method [9] with glucose as standards.

One unit of amylase activity was defined as the amount of the enzyme that librated 1 µmol/min of reducing sugar as glucose from soluble starch under the standard assay conditions.

RESULT & DISCUSSION:

The sugarcane is a cash crop and plays an important role in the national economy. The sugarcane waste (Bagasse & Leaves) was hydrolyzed with 0.6N H_2SO_4, HCl, NHO_3, $HClO_4$, NaOH & KOH to fermentable sugar and were used for the cultivation of *Penicillium expansum* and production of amylase. It has been reported in the literature that the yield of fermentatable sugarcane varies from acid to acid and carbon-to-carbon source in the process of hydrolysis [10-12]. The rate of amylase production increases with the increase of growth period and reaches maximum at 168 and 144 hours when penicillium expansum was grown on sugar cane bagasse and leaves treated with 0.6N H_2SO_4 and filtrate was supplemented with mineral medium as shown in Fig. 1 & 2. During fermentation period, the pH of the culture medium remains nearly constant while the concentration of total sugar in the medium continuously decreases with the increase of incubation time period.

Maximum production of amylase was achieved at 144 hours in case of sugar cane bagasse treated with 0.6N HNO_3

Fig. 1 **Effect of 0.6N H_2SO_4 pretreated bagasse as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted 200 rev/min with initial pH 6.0 at 28 ± 2°C.**

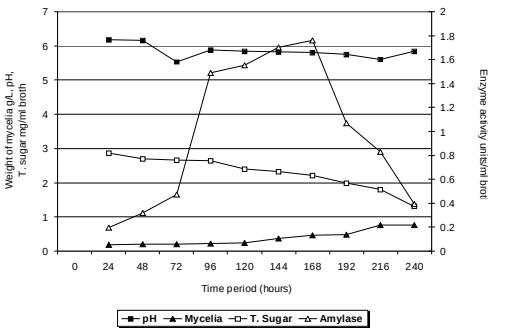


Fig. 2 **Effect of 0.6N H_2SO_4 pretreated sugarcane leaves as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.**

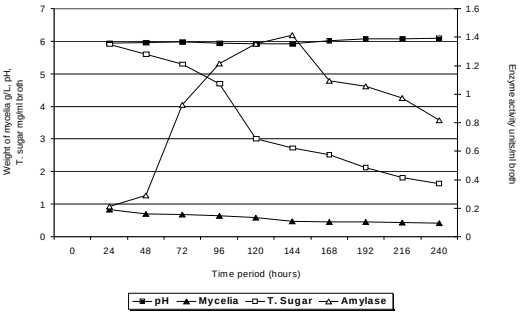
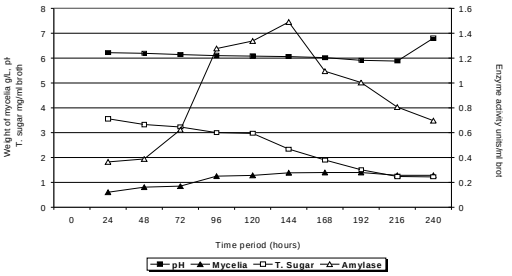
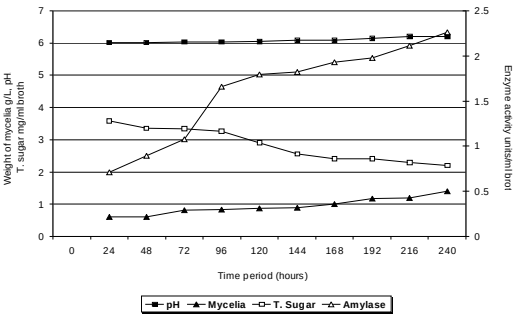


Fig. 3 Effect of 0.6N HNO_3 bagasse as a carbon source on the growth of Penicillium expansum and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with intial pH 6.0 at 28 ± 2°C.



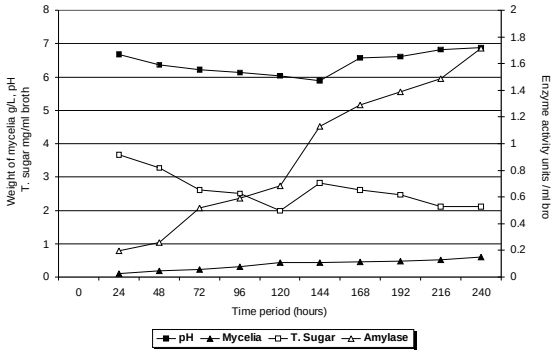
(Fig. 3) while the rate of amylase production increases with the increase of fermentation period in case of sugar cane leaves treated with 0.6N HNO_3 and the filtrate was incorporated with mineral medium for the growth of penicillium

Fig. 4 Effect of 0.6N HNO_3 pretreated sugarcane leaves as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.



expansum as shown in Fig. 4. The pH of the culture medium slightly increases during the production of amylase but the

Fig. 5 Effect of 0.6N HCl pretreated bagasse as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min initial pH 6.0 at 28 ± 2°C.

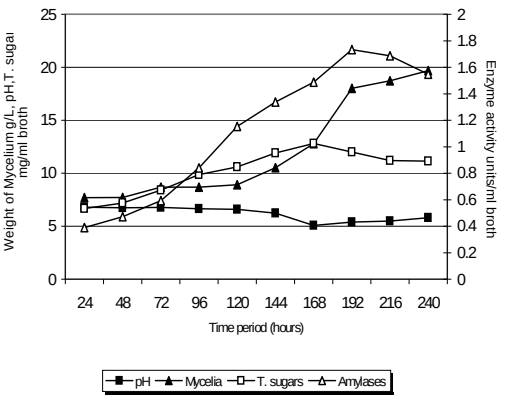


concentration of total sugar in the medium decreases through out the study period. However, mycelial biomass weight of Penicillium expansum increases with the increase of growth time period.

Fig. 5 and 6 represents the data of amylase production by penicillium expansum grown on mineral medium containing the hydrolysate of sugar cane bagasse and sugar cane leaves hydrolyzed with 0.6N hydrochloric acid. It is clearly seen from the data that the rate of amylase production

increases with increase of incubation period up to 240 and 192 hours in case of sugar cane bagasse and leaves respectively. Final pH of culture medium and mycelial biomass were increasing order through out study period.

Figure 6Effect of 0.6N HCl pretreated sugarcane leaves as a carbon source on the growth of Penicillium expansum and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev./min with initial pH 6.0 at 28 ± 2°C.



The production of amylase increases with the increase of incubation time period up to 120 hours and then decreases by penicillium expansum when grown in 0.6N $HClO_4$ pretreated sugar cane bagasse and leaves. The concentration of

sugar in the culture medium decreases with the growth of penicillium expansum through out study period and the results are presented in Fig. 7 and 8. A significant increase was not noted in the final pH of the medium and mycelial biomass.

Fig. 7 Effect of 0.6N $HClO_4$ pretreated bagasse as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28± 2°C.

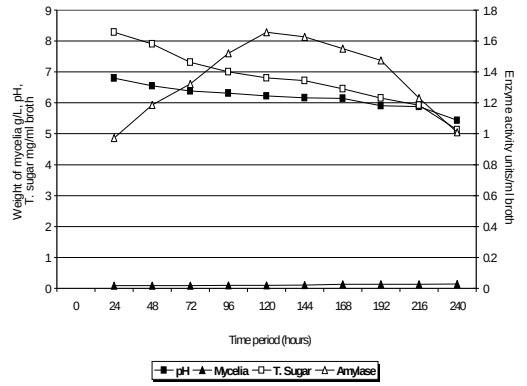


Fig. 8 Effect of 0.6N $HClO_4$ pretreated sugarcane leaves as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28±2°C.

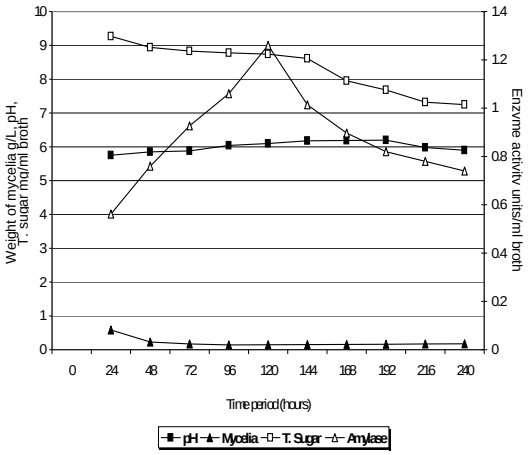


Figure 9 and 10represents the data of Amylase production by penicillium expansum grown on 0.6N NaOH treated

10 Effect of 0.6N KOH pretreated bagasse as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.

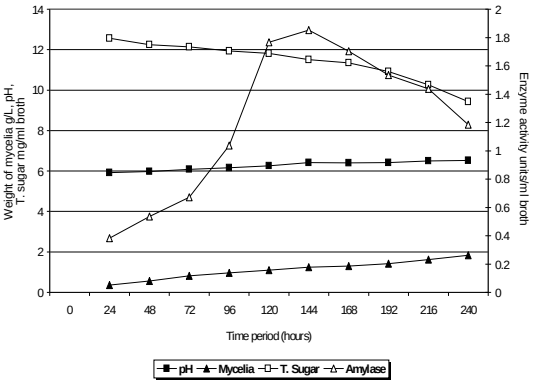
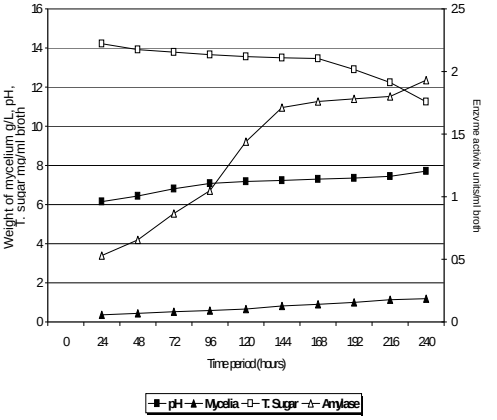
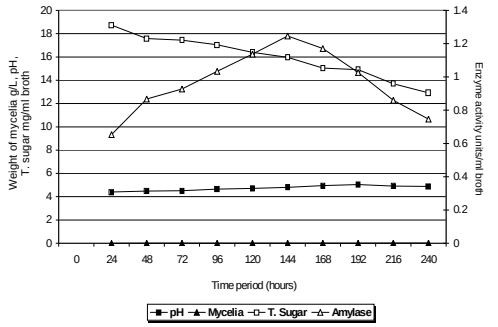


Fig. Effect of 0.6N NaOH pretreated bagasse as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.



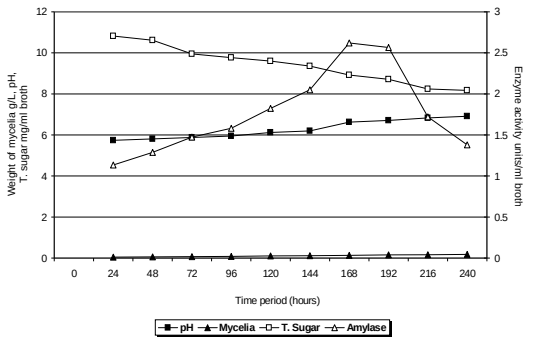
sugar cane bagasse and leaves. Maximum amylase production 2 and 1.2 units/ml were achieved at 240 and 144 hours and then declined in case of sugarcane bagasse and leaves hydrolysate medium. Mycelial biomass and final pH of culture medium slightly increases while the concentration of reducing sugar decreases with the increase of incubation period.

Fig. 9 Effect of 0.6N NaHO pretreated sugarcane leaves as a carbon source on the growth of *Penicillium expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.



Amylase production by penicillium expansum grown on 0.6N KOH pretreated sugarcane bagasse and leaves increases with the increase of time up to 120 and 168 hours as depicted in Fig. 11 and 12. the greater amount of amylase (2.7 units/ml) was produced in sugar cane leaves in comparison to sugar cane

Fig 11 Effect of 0.6N KOH pretreated sugarcane leaves as a carbon source on the growth of *Penicillim expansum* and production of Amylases. Culture was incubated in cooled orbital shaking incubator adjusted at 200 rev/min with initial pH 6.0 at 28 ± 2°C.



bagasse (1.8 units/ml). Final pH of culture medium and mycelial biomass increases with the increase of time period.

The study was carried out to hydrolyze the sugarcane waste (bagasse & sugarcane leaves) to fermentable sugar by 0.6N H_2SO_4, HCl, NHO_3, $HClO_4$, NaOH & KOH. The results are shown in Fig.1-10, the maximum production of amylase achieved 1.7632/1.4136 Units/ml broths

at 168 & 144 hours by *Penicillium Expansum* grown as 0.6N H_2SO_4 pretreated sugarcane bagasse & leaves. Respectively, at 144 and 240 hours in case of 0.6N HNO_3 pretreated bagasse & sugarcane leaves produced 1.4896 and 2.2648 units/ml broth, at 240 and 120 hours in case of 0.6N HCl pretreated bagasse & sugarcane leaves produced 1.7176 and 2.416 units/ml broth, at 120 hours in case of 0.6N $HClO_4$ pretreated bagasse & Sugarcane leaves achieved

1.6568 and 1.260 units/ml broth, at 240 and 144 hours 0.6N pretreated bagasse & sugarcane leaves produced 1.9304 & 1.2464 units/ml broth 240 and 144 hours in case of 0.6N NaOH pretreated bagasse & sugar can leaves produced 1.9304 and 1. 2464 units/ml broth at 144 and 168 hours in case of 0.6N KOH pretreated bagasse & sugarcane leaves produced 1.852 and 2.6208 units/ml broth.

The present work shows that it is feasible to use agro waste for the production of amylase by Penicillium expansum. This newly isolated fungus is able to produce extra cellulose amylase during submerged batch wise fermentation.

It is observed that there is no relationship between time period of growth and Amylase synthesis because of the nature of carbon source, pH of the culture medium, composition of culture medium & species of the organism play important role for the synthesis of enzymes. [13-14]

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