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Enzymatic Hydrolysis of *Mangifera indica* Saw Dust and its Use as Potential Feedstock for the Citric Acid production and Single Cell Rotein

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Abstract: Enzymatic hydrolysis of cellulose obtained from *Mangifera indica* saw dust after high pressure steaming and alkali treatment, was carried out by the partially purified cellulase and β-glucosidase obtained from *Trichoderma viride* and *Aspergillus wentii*, respectively. The incubation period 48 h, temperature 50 ^{0}C and 30 units (IU) of cellulase per gram of cellulose fiber were sufficient for 67 % Saccharification at pH, 4.8. Below and above pH-4.8 the percent hydrolysis of cellulose declined sharply. Supplementation of *Aspergillus wentii,* β-glucosidase to *Trichoderma viride* cellulase, nearly 80 % of cellulose was hydrolyzed in 48 h compared to 54.6 % without β-glucosidase under optimum condition. The enzymatic hydrolysate was further used for the production of yeast biomass and citric acid fermentation. *Mangifera indica* residue which can be used as a substrate for enzyme production which reduces the cost of enzyme production and enzymatic conversion of carbohydrate part of *Mangifera indica* into fermentable sugar.

Key words: *Trichoderma viride*, *Aspergillus wenti*, β-glucosidase, *Mangifera indica*, cellulase, saw dust.

Introduction

The lignocellulose tissues of higher plants, including soft and hard wood are the major repository of photosynthetic energy and renewable organic matter on the earth. The stature of the lignocellulosics is based on three main components: cellulose, hemicellulose and lignin, offers tremendous biotechnological potential for use as substrate in bioconversion. The recent thrust in bioconversion of agricultural and industrial wastes to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by fungi and bacteria[1]. Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particular from agro-allied industries such as breweries, paper-pulp, textile and timber industries. The plant biomass regarded as "wastes" are bio-degradable and can be converted into valuable products such as bio-fuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients [2].

The polysaccharide component of lignocellulose includes cellulose and hemicelluloses, which amounts to 60-80 % (w/w) of the total system. In addition to cellulose hemicelluloses and lignin plant cell wall contains extraneous components including extractives and non-extractives. Extractives consist of fats, waxes, tannins, resins, etc. The non-extractives of extraneous components mainly consist of inorganic com-ponents such as silica, carbonates, oxalates etc [3]. Cellulose has

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enormous potential as a renewable source of energy [4]. A great variety of fungi and bacteria can fragment these macromolecules by using hydrolytic or oxidative enzymes and use as a carbon source. Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds [5].

Trichoderma spp. is favored and promising sources of cellulolytic enzyme [6], but its produce relatively low amount of β-glucosidases [7]. Therefore its, enzymes system produces cellobiose instead of glucose as major product in hydrolysates. However, supplementation to the enzyme complex with β-glucosidases from different source would enhance the rate of hydrolysis of cellulose in to fermentable sugars and improves cellulose digestibility. The purpose of the present study was to utilize the culture filtrate of *Trichoderma viride* and *Aspergillus wentii* produced in batch fermentation and containing high specific activity cellulases and β-glucosidases for enzymatic hydrolysis of *M. indica* saw dust to fermentable sugars.

Material and Methods

Enzyme source

The enzyme source was partially purified culture filtrates of *Trichoderma viride* and *Aspergillus wentii*. The medium composition was similar to that used by Mendel & Weber [8], with the slight alternation, instead of cellulose and peptone, 1 % glucose was used as a carbon source in inoculum media. However, in case of enzyme production medium the carbon source was pretreated *Mangifera indica* saw dust. The fermentation was carried out in 3L capacity Bioengineering laboratory fermenter. Tween-80 (0.03 % v/v) and $MgSO_4.7H_2O$ (0.03 % v/v) were used in batch culture of *T. viride* and *A. wentii* to enhance the production and excretion of extracellular cellulases and β-glucosidases, respectively.

Enzyme assay

Cellulase activity was assayed using Whatman no.1 filter paper and carboxy-methyl cellulose (CMC) by the method of Ghose [9], β-glucosidase activity was assayed using both p-nitrophrnyl-b-D-glucopyranoside and cellobiose as substrate [10]. Total reducing sugar was measured by dinitrosalicylic acid (DNS) method [11]. The activity was expressed as international unit (IU/ml).

Pretreatment of lignocellulosics

Saw dust of *Mangifera indica* was procured from local market and washed with water to remove soluble impurities. The saw dust of particle size in the range of 20 mesh or less was suspended in water to obtain 10-15 %, (w/v) suspension and subjected to high pressure steaming at 350 psi for 30 min. The hemicellulose components rendered soluble in water. The hemicellulose free residue was then subjected to aqueous alkali treatment. The insoluble material was separated by filtration, dried and used as a pretreated lignocellulose substrate for enzymatic hydrolysis.

Enzymatic treatment

Enzymatic hydrolysis of pretreated *Mangifera indica* saw dust was performed at pH 4.8, 50°C and 2.5 % (w/v) substrate concentration for 20 h. After stipulated time the reaction was stopped by immersing the reaction vessel in boiling water bath for 5-10 min. The residual material was removed by centrifugation. The supernatant was decanted and reducing sugar was expressed as glucose. The procedure scaled up to 5 L.

Cultivation of yeast on enzymatic hydrolysate of *Mangifera indica* saw dust

The lignocellulosic hydrolysate containing sugar concentration of 2 % (w/v) added to carbon free Czapex Dox medium. It was supplemented with 0.1 % peptone. The medium was sterilized at 121°C 15 psi for 15 min and inoculated with yeast cells (*Saccharomyces cerevisiae*) for production of biomass incubated at 30°C for 20 h on rotatory shaker at 150 rpm. At the end of incubation period the cell mass was separated by centrifugation, washed with water and dried at 80° C overnight, cooled and weighed.

Production of citric acid

The medium used for the growth of *A. niger* inoculum and citric acid production was composed of enzyme pretreated cellulosic hydrolysate of *Mangifera indica* saw dust. The concentration of glucose in hydrolysate of *M. indica* saw dust was adjusted to 10 % (w/v) and further supplement

with NH_4NO_3 (0.223 %, w/v), K_2HPO_4 (0.1 %, w/v), and $MgSO_4.7H_2O$ (0.023 %, w/v). The initial pH of the medium was adjusted to 2.2 with 1N HCl. Total citric acid was mea-sured by the method of Marrier and Boulet [12].

Results and discussion

Enzymatic hydrolysis of Mangifera Indica saw dust

The cellulase enzyme prepared from *Trichoderma viride* was used to study the saccharification of the cellulose fiber from *Mangifera indica* saw dust (Fig. 1) over a period 48 h. It was found that the rate of saccharification reaches to maximum in 40 h regardless of the substrate concentration. However, concentration of cellulose was found to have a profound influence the percentage of saccharification. The maximum hydrolysis was achieved at 2.5 % (w/v) cellulose concentration where as at 10 % (w/v) cellulose concentration the saccharification of cellulose was decreased by 30-35 %. This shows that at lower concentration the substrate is easily accessible to the enzyme than at the higher substrate concentration. This was further supported by the fact that at 2.5 % (w/v), cellulose concentration maximal hydrolysis was attained in less than 24 h as compared to 40 h or more at higher concentration. These results suggest that the substrate enzyme ratio may play an important role in efficient degradation of the cellulose (Fig. 2) shows the percent Saccharification of *Mangifera indica*

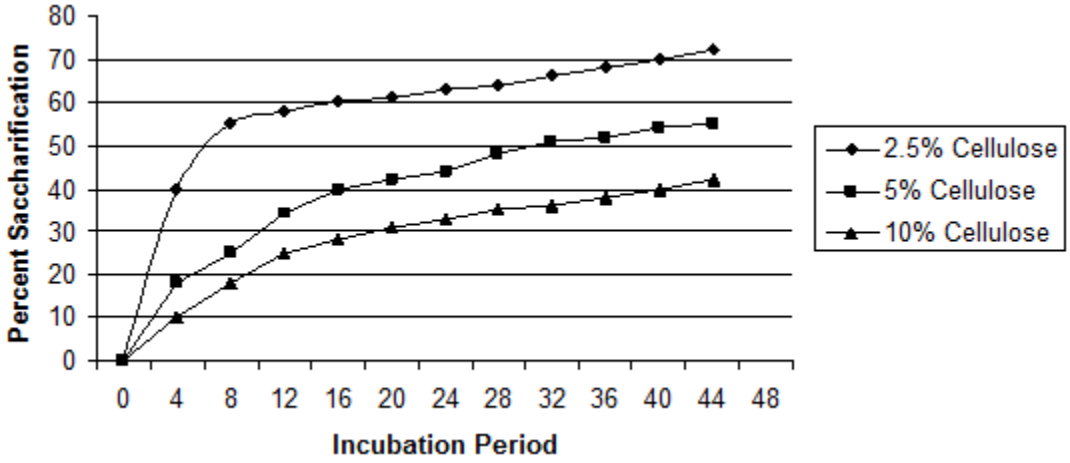

Fig. 1. Hydrolysis of pretreated *Mangifera indica* saw dust by *Trichoderma viride* cellulase

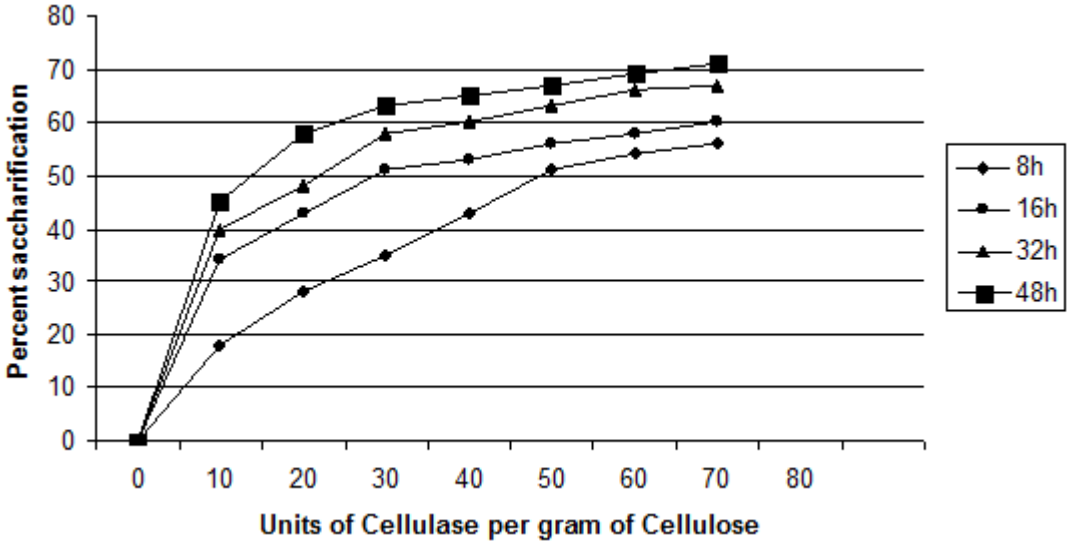

Fig. 2. Hydrolysis pretreated *Mangifera indica* saw dust as a function of cellulose concentration of *Trichoderma viride* at different period of incubation

saw dust as a function of cellulose ratio. It was found that in first 8 h. of digestion the enzyme: cellulose ratio has profound effect on the rate of hydrolysis of cellulose. The hydrolysis require large mount of enzyme (70 unit/g. of cellulose) achieve 60 % Saccharification in 8h. Furthr if the incubation carried out for 16h or longer period, 20-30 units of cellulose per gram of cellulose fiber were sufficient for 65-70 % Saccharification of cellulose. Thus, by increasing the enzyme concentration from 30 units/g. cellulose to 70 units/g. cellulose, time require for achieving maximum Saccharification 70 % was reduced from 48 h. to just 16 h. (Fig. 3). Since the amount of cellulase was a limiting factor, incubation for 40 h. using 20-30 units of enzyme per gram of cellulose was preferred. Under these condition the hydrolysis of pretreated *Mangifera indica* saw dust was about 70 % compared to only 46 % hydrolysis of the avicel microcrystalline cellulose (Fig 4). It shows that crystal linty of the *Mangifera indica* saw dust was greatly reduced after the high pressure steaming followed by delignification by alkali extraction.

The saccharification of the cellulose fiber of *Mangifera indica* was carried out at pH range of 3.0 - 6.0 and temperature range of 40° C to 60°C. The results are shown in Table 1 and 2. It was observed that maximum enzymatic activity by *Trichodrema viride* cellulase occur at pH 4.8. Below and above this pH, the percent hydrolysis declined sharply. Moreover after 20h of digestion

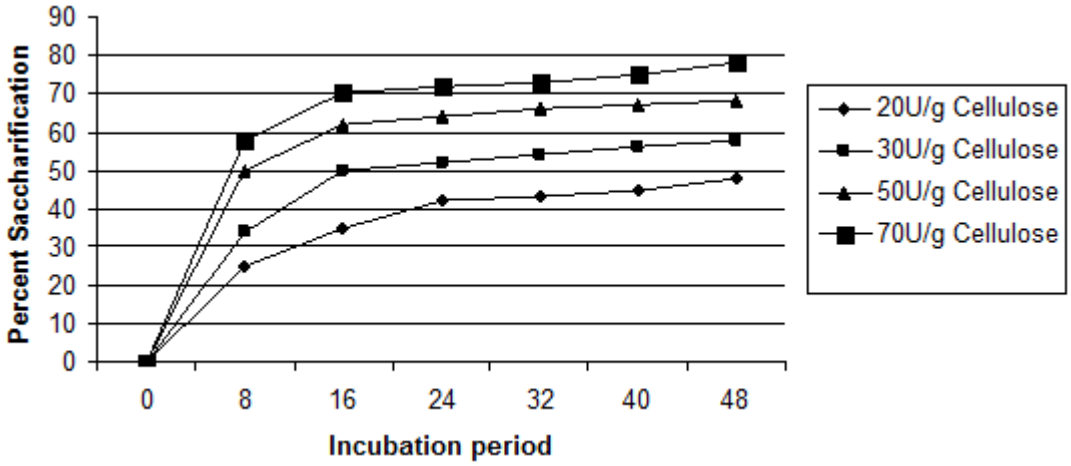

Fig. 3. Hydrolysis of *Mangifera indica* saw dust as a functional of time with different concentration of cellulase of *Trichoderma viride*

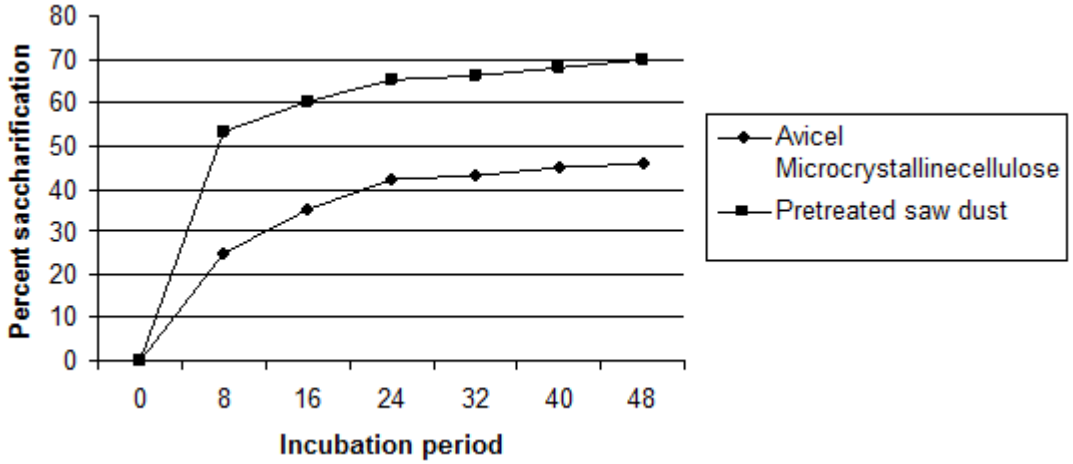

Fig. 4. A comparison of enzymatic hydrolysis of pretreated *Mangifera indica* saw dust and avicel microcrystalline cellulose

Table 1. Effect of pH on Saccharification of the pretreated *Mangifera indica* saw dust by *Trichoderma* viride cellulase

pH	Incubation period (h) 20 Saccharification (%)	36 Saccharification (%)
3.0	12.5	18.0
3.5	22.4	27.5
4.0	32.5	34.0
4.5	43.0	51.5
4.8	62.0	68.0
5.0	60.5	64.8
5.5	41.5	48.0
6.0	24.0	33.5

Table 2. Efffect of temperature on Saccharification of the pretreated *Mangifera indica* saw dust by *Trichoderma viride*

Temperature (°C)	Incubation period Saccharification (%) 8h	20h	36h	48h
40	27.4	33.1	36.2	39.7
45	43.9	52.1	58.4	64.1
50	51.7	63.0	67.5	69.0
55	45.1	49.1	56.2	61.4
60	25.1	32.2	37.3	42.2

there was only marginal increase in the saccharification of cellulose. The optimum temperature for the enzymatic hydrolysis was found to be 50°C. Similarly the effect of pH, only a marginal increase in the percentage of cellulose hydrolysis was noticed when incubation longer than 20 h was performed.

For the efficient enzymatic digestion of cellulose, the high specific activity β-glucosidase from *Aspergillus wentii* (1.2 units /mL) was employed along with the *Trichoderma viride* cellulase. Results shown in Table 3 clearly demonstrate that supplementation of cellulose with β-glucosidase markedly increased the percent Saccharification of cellulose as greatly shortened the incubation period. In presence of β-glucosidase nearly 80 % (w/w) cellulose was hydrolyzed by *Trichoderma* cellulase in 48h compared to only 54.6 % without β-glucosidases under similar conditions. This may be attributed to the fact that cellobiose was produced during digestion of cellulose by the action of cellulase activity was greatly inhibited by cellobiose itself. Thus, for the efficient enzymatic digestion of cellulose it is essential that level of cellobiose is kept as low as a possible. The hydrolysis of *Mangifera indica* saw dusts by cellulase complex are in complete agreement with the accepted model for the enzymatic hydrolysis of cellulose. In the present study, simultaneous hydrolysis of cellobiose by β-glucosidase results in an increased rate of cellulose digestion and release of fermentable sugars.

SCP production from the enzymatic hydrolysate offers a potential substrate for the bioconversion into an improved animal feed and human food. Among the SCP obtained from the lignocellulosic waste as the main growth media, *Saccharomyces cerevisiae*, *Trichoderma ressei* and *Kluveromyces* top the list [12]. Besides this, organic acid are some of the product of ligninolytic residues fermentations via environmentally friendly integrated processes. Volatile fatty acids including acetic

Table 3. Effect of *Aspergillus wentill* β-glucosidase supplementation on hydrolysis of cellulose by *Trichoderma viride* cellulase

β-glucosidase concentration (units/ml)	Incubation period (h) 8 h Residual sugar (mg/ml)	8 h Saccharification (%)	16 h Residual sugars (mg/ml)	16 h Saccharification (%)	24 h Residual sugars (mg/ml)	24 h Saccharification (%)	48 h Residual sugars (mg/ml)	48 h Saccharification (%)
0.0	6.5	23.4	10.16	36.6	12.6	45.6	15.16	54.6
0.4	9.0	32.4	13.0	46.8	16.3	58.8	18.50	66.6
0.8	11.0	39.6	15.3	55.8	18.0	64.8	21.56	77.6
1.2	12.3	44.4	17.3	62.4	20.1	72.6	22.30	80.4

acid, propionic acids and butyric acid are produced from a wide range of LSW such a cereal hulls [13,14,15], bagasse residues [16], food waste [17] and sisal leaf decortications residues [18]. The results of the present study are inagreement with the work of the previous workers [19,20,21,22,23,], where they observed the maximum rate of hydrolysis in 4-8 h of enzyme substrate incubation [24,25,26,27]. The decline in hydrolysis rate could be due to the increasing resistance of the substrate during the course of hydrolysis or other factors [20,24,25].

The enzymatic hydrolysates of *Mangifera indica* saw dust was used as carbon source for growing yeast (*Saccharomyces cerevisiae*) and for citric acid fermentation by *Aspergillus niger* (Fig. 5) shows the cultivation profile of *Saccharomyces cerevisiae* on saw dust hydrolysate. It was observed that the production of yeast on enzymatic hydrolysate enzymatic hydrolysate of *Mangifera indica* saw dust comparable to that of the standard fermentation medium in which 2 % dextrose was present as carbon source. The process was scaled up to 10 L. to obtain about 1 Kg yeast in 24 h.

The result of citric acid production by *Aspergillus niger* utilizing hydrolysate containing 10 % sugar as glucose is shown in **(**Fig 6.).

It was observed that sugar was converted to citric acid and the maximum production of 58 g/L was obtained after 10 days of incubation in submerged batch fermentation. In addition, the profile of citric acid production with enzymatic hydrolysate was found to be comparable with glucose as a substrate for the citric acid production.

Mangifera indica saw dust may be used as cheap substrate for citric acid production. The hydrolysis of the *Mangifera indica* saw dust by cellulase complex is in complete agreement with accepted model for enzymatic hydrolysis of cellulose [19]. The result appears to be quite promising and has the potential of being developed into an environmentally sound biotechnological application for sustainable development. Further the process holds tremendous prospects and favourable socio-economics impacts.

Conclusion

The purpose of present study was to explore the possibility of using lignocellulosic waste for the production of fermentable sugars which in turn

would provide cheap substrates for the production of the value added products i.e. single cell protein, citric acid fermentation, etc.

The hydrolysate obtained was used directly for the production of single cell protein and also for the citric acid production by fermentation using *Saccharomyces cerevisiae* and *Aspergillus niger*, respectively.

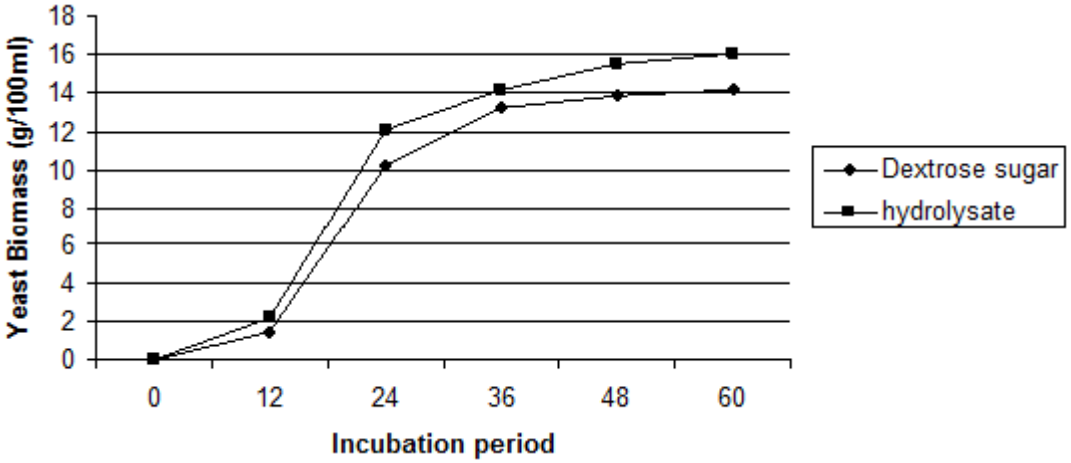

Fig. 5. Production profile of a single cell protein(*Saccharomyces cerevisiae*) on hydrolysate (2% reducing sugar) of *Mangifera indica* saw dust

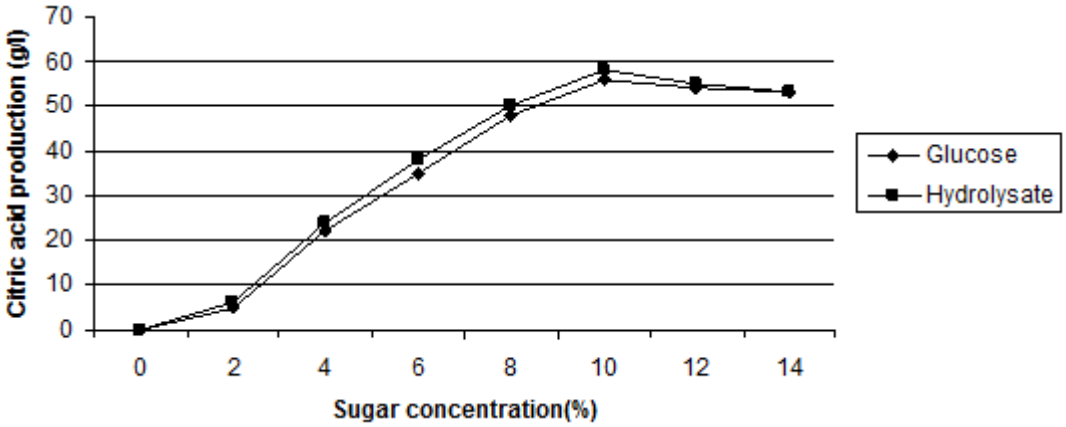

Fig. 6. Citric acid production utilizing different concentration (reducing sugar) of enzymatic hydrolysis of *Mangifera indica* saw dust using *Aspergillus niger*

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