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COMPARISON OF ACID AND ALKALI CATALYTIC EFFICIENCY DURING ENZYMATIC SACCHARIFICATION OF CORNCOB AND LACTIC ACID PRODUCTION

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Corncob is an agriculture waste found to consist of 38.9% cellulose and 43.4% hemicellulose on dry solid (w/w %) basis and thus considered a potential source for fermentable sugars. These sugars can potentially be extracted through combined chemical pretreatment and enzymatic saccharification. The present study is aimed to optimized the pretreatment process by using variety of alkali and acids includingH_2SO_4, HCl, H_3PO_4, $C_2H_2(COOH)_2$, CH_3COOH, NaOH, NH_3OH and H_2O as a catalyst at different temperatures and reaction time ranged from 100–130°Cand 0.5-5h, respectively. The pretreated biomass was then hydrolyzed with cellulose enzyme (Accellrase 1500) for production of fermentable sugars. The H_3PO_4 and $C_2H_2(COOH)_2$ treated material showed 88+1% hydrolysis yield while NaOH and NH_3OHyielded>95.0%. The order of catalytic effectiveness on enzymatic hydrolysis remained as NaOH > NH_3OH > H_3PO_4 > $C_2H_2(COOH)_2$ > HCl > H_2SO_4 > CH_3COOH > H_2O. Acids also bequeath 30-42gL^{-1} of reducing sugars during pretreatment step, while alkali degrades them. Pure glucose obtained from H_2SO_4 and HCl treated materials while mixture of xylose and glucose was obtained from alkali treated material. The resulting sugars obtained from enzymatic hydrolyzate were successfully fermented into lactic acid by using *L. acidophilus*, with above 90.0% conversion efficiency and 0.78 gL^{-1}h^{-1} lactic acid productivity obtained from maleic acid treated enzymatic hydrolyzate.

Keywords: Corncob, Cellulase, Maleic acid, Pretreatment, Lactic acid, *Lactobacillus acidophilus*

INTRODUCTION

Agricultural residue is gaining much importance in these days because of its abundance, low cost, whole year decentralized availability for the biological production of industrial chemicals such as glucose, furfural, hydroxymethyl furfural, levulenic acid, lactic acid, acetic acid, propionic acid and fuels (Wyman *et al.,* 1992; Thomson *et al*., 2009). The lignocellulosic material contains about 75-80% of hemicellulose and cellulose which are the source of monomeric sugars required for fermentation. Lignocellulosic substrate with recalcitrant nature is difficult to hydrolyze into fermentable sugars. The advancement in genetic engineering regarding the exploration of new varieties of microorganisms used for production of cellulase enzyme make it possible to hydrolyze such materials. Enzymatic hydrolysis of cellulosic material is the most promising approach to get high yield of monomeric sugars as compared to concentrated acid or dilute acid hydrolysis which is vital for the economic success of described method (Hinman *et al.,* 1992; Lynd *et al.,* 1996).

The successful enzymatic hydrolysis of biomass can be performed with the help of suitable pretreatment method which will enable the enzyme to hydrolyze the cellulose and hemicellulose by removing the lignin and diminishing the crystallinity of cellulose (Galbe and Zacchi 2002; Zhue & Pan 2010; Kumar *et al.,* 2009).These methods include acid and alkaline pretreatment, auto hydrolysis, steam explosion, ammonia fiber explosion, carbon dioxide & sulphur dioxide explosion, wet oxidation and organic solvent (Jiele *et al.,* 2010; Alizadeh *et al*., 2005; Sun and Cheng 2005). For cost effectiveness and process efficiency, pretreatment of biomass was executed with mineral acids such as sulphuric acid, hydrochloric acid or nitric acid (Patel *et al.,*1993; Li *et al.,* 2008; Laopaiboon *et al.,* 2010) and alkali like NaOH or NH_3OH (Zhao *et al.,* 2008) at different temperatures for different times. Although a number of investigations have been performed for corncob and reported by various researchers such as pretreatment with steam (Garrote *et al*., 2001), phosphoric acid (Satimanont S. *et al.,* 2012), sodium hydroxide (Ouyang J. *et al.,* 2009) but none of these provided comparative description of the pretreatment conditions for production of maximum fermentable sugars from corncob and their use in lactic acid production.

Maize is a major food crop after wheat and rice in Pakistan with annual production of 3760×1000MT during year 2010-2011, which provides raw material to industry and feed to livestock and poultry as well (Khaliq *et al*., 2004). It contributes more than 10.0% of all agricultural production. The corn crop contains corn and corn stover in equal proportions, latter has 15-20.0% of corncob (Hanway *et al.,* 2007; Sokhansanj *et al.,* 2002) which is available for further use. Limited studies have been reported on the use of corncob as a source for bioethanol or lactic acid. The aim of this work was to determine the suitable catalyst and optimum pretreatment condition to maximize the hydrolysis yield of cellulose present in corncob. During optimization of pretreatment conditions, comparative effect of different catalysts on biomass reduction, production of reducing sugars during pretreatment and enzymatic step was recorded for obtaining maximum benefit from the process through accumulative yield of sugars in both stages of hydrolysis. After this the utility of these sugars were studied through the production of lactic acid.

MATERIAL AND METHOD

Chemicals**:** Two types of enzymes, ACCELLERASE 1500 and OPTIMASH™ BG were obtained from Genencor International Inc. *Lactobacillus acidophilus ATCC 4356* was obtained from Microbiologies, Inc. USA (Lot#256053). All reagents used in this study were of analytical grade. Concentrated sulphuric acid, phosphoric acid, hydrochloric acid, maleic acid, sodium hydroxide, ammonia and D(+)-Glucose obtained from Panreac, D(+)-Xylose (GPR) from BDH Ltd (England) and L-(+)-Arabinose was obtained from Sigma Aldrich. α-naphthol was purchased from Merck (Darmstadt, Germany). 3, 5-Dinitrosalicylic acid and analytical grade phenol was procured from Fluka Chemie.

Corncob**:** Corncob was collected from a local farmer from Qasoor district, Punjab Pakistan. After collection, it was dried in sunlight for five days, cut into small pieces and ground by using pulveriser equipped with 150 mesh steel sieves. The dried powdered material was then stored at room temperature.

Pretreatment of corncob**:** The powdered dry mass was used for pretreatment with different reagents. Fifty grams of corncob powder were mixed with 1.0% solution of sulphuric acid, hydrochloric acid, phosphoric acid, acetic acid, maleic acid, sodium hydroxide and ammonia, in 1: 6 w/v ratios, in a 500 ml flask separately. The flasks were autoclaved (CL-40L, ALP Co, Ltd. Tokyo Japan) at different temperatures ranging from 100-130°C at pressure 0.11MPa for different time intervals ranging from 0.5 to 5.0h. The flasks were cooled and contents were filtered using Whatman filter paper No. 1 to extract the cellulosic residue from hydrolyzate. The pH of acid hydrolyzate was adjusted at 5.0 -6.0 with the help of sodium hydroxide, filtered and then analyzed the reducing sugars with the help of DNS method (Ghose, 1987). The residue was washed with distilled water for several times to bring the pH at 4.8 and then dried at 70.0°C for overnight and weighed. The effect of pretreatment on the substrate was shown in Fig.1.

Figure 1. a:corncob before treatment, b:maleic acid treated, c:sulphuric acid treated, d:phosphoric acid treated, e: NaOH treated

Enzymatic hydrolysis of pretreated material**:** Enzymatic hydrolysis was done by using the solid residue obtained after every treatment with commercial enzyme (Accellrase & Optimash BG) obtained from Genencor International. Accellrase 1500 has multiple enzyme activities and is effective for hydrolysis of cellulose, hemicellulose and ß-glucans. OPTIMASH™ BG is a mixture of Xylanase and ß-Glucanase which was used together with ACCELLERASE 1500 to enhance the enzymatic hydrolysis efficiency of hemicellulose. The enzymatic hydrolysis was carried out in 250.0mL glass flask. ACCELLERASE 1500 and OPTIMASH™ BG (0.2 & 0.1 mLg^{-1} dry wt. of corncob, respectively) were used for hydrolysis. Five grams of pretreated dry mass of corncob were added in each flask separately. The pH of the reaction mixture was set at 4.8 by adding 100.0 mL of 0.1M acetate buffer solution. The flasks were kept in orbital shaker for 48.0h at 50.0°C with 160 rpm. After regular time intervals (12.0h), samples were taken from each flask and kept in boiling water for 5.0 min to inactivate the enzyme. Each sample was filtered on Whatman filter paper No.1 and subsequently analyzed. Each experiment was performed in triplicates.

Lactic acid fermentation**:** Lactic acid was produced from the lactic acid producing bacteria Lactobacillus *acidophilus,* homo fermentative bacteria (Ki Yong *et al.,* 2001). The lactobacillus strain was maintained on potato dextrose agar slants before use. The basal medium contains the 10.0 gL^{-1} yeast extract, 2.0 gL^{-1} $(NH_4)_2HPO_4$, 0.1 gL^{-1} $MnSO_4$ and 30.0 $gL^{-1}$of glucose. Inoculum was prepared by transferring cells into 100.0 mL Erlenmeyer flask containing 50.0 mL of growth medium containing 30.0 gL^{-1} of glucose and incubated at 37.0°C for 12.0h (Young *et al.,* 2006) in rotary incubator shaker. After two consecutive transfers to fresh medium, this was used to inoculate the fermentation medium. Sugar solution containing glucose and xylose obtained from the enzymatic hydrolyzate of corncob at optimized conditions in presence of different catalysts were supplemented with 2.0 gL^{-1} $(NH_4)_2HPO_4$, 0.1 gL^{-1} of $MnSO_4$ and 10.0 gL^{-1} yeast extract. For lactic acid production inoculum to sugar solution ratio of 1:20 was used. Samples, for glucose and lactic acid analysis, were taken at specific intervals during 72.0h fermentation process.

Analysis of lignin & hemicellulose, cellulose, reducing sugars and lactic acid: The amount of extractives, hemicellulose, cellulose and lignin before and after pretreatment of the corncob was determined by method outlined in Idrees M. *et al*., (2013).Total reducing sugar in hydrolyzate was determined through DNS (3, 5-dinitrosalicylic acid) method. One ml of sugar solution was mixed with equal volume of DNS reagent and incubated at 80°C for 5.0 min. After color development, put it in ice cooled water to intensify the color. The color intensity of each sample was measured at λ=546 nm with double beam spectrophotometer (Cecil Aquarius CE 7200).The quantity of sugar was calculated through standard curve obtained from different concentrations of glucose (R^2=0.9832). Thin layer chromatography (TLC) was used for identification of sugar contents in hydrolyzate obtained from pretreated media, enzymatic hydrolyzate and fermentation media, method used by Hyeon *et al.,* (2009). Kieselgel 60F 254 (Merck) plats having size 20×20 cm was used as stationary phase. A solvent mixture consisting of acetonitrile and water (85:15 v/v) was used as eluting solvent, visualization of spot was done by soaking plats in 0.5% α-naphthol made in acidic solution of ethanol (5.0%H_2SO_4). After soaking, plats were dried at 80°C for 5.0 min in hot oven. The sugar yield was calculated on pretreated solid biomass used for enzymatic hydrolysis by using the following equation used by Dedsuksophon *et al.,* (2011).

$$\text{Sugar Yield (\%)} = \frac{100(\text{sugar produced during hydrolysis})}{(\text{gram of biomass feedstock})}$$

The quantity of lactic acid was determined in the fermentation media with the help of reversed phased HPLC, method used by Bai *et al.,* (2000). Sample was centrifuged at 2000 rpm for 5min and filtered with membrane filter (Sartorius SM 11606).After filtration, clear solution was directly used for HPLC analysis. LC-20AT (Shimadzu) system was used having SMA-C18 column with size 4.6×250mm of SMT coupled with a UV variable wavelength detector (SPD-M20A, Shimadzu) which was set at 210nm. Elution was carried out with the help of 0.01M phosphoric acid having pH 2.5 and flow rate of 1.0ml/min at room temperature. Concentration of lactic acid in each sample was determined from the calibration curve obtained between lactic acid conc. and their peak area. The retention time of lactic acid (t_R) was 2.4 min.

RESULTS AND DISCUSSION

Corncob was considered as a prospective source for the production of fermentable sugars which could be converted into fuel and bulk chemicals (Ming *et al.,* 2007; Mirua *et al.,* 2004 & Moldes *et al.,* 2006) due to its high cellulosic content (55-75%) (Sun *et al.,* 2002). Before mining of these monomeric sugars through enzymatic hydrolysis the crystallinity of cellulose and hemicellulose was decreased by pretreating it with H_2SO_4, HCl, H_3PO_4, $C_2H_2(COOH)_2$, CH_3COOH, NaOH, NH_3OH and H_2O at different conditions of temperature (100-130°C) and residence time(0.5-5.0h). The composition of corncob before and after pretreatment was shown in Table 1 and physical appearance was shown in fig.1. The amount of cellulose, hemicellulose and lignin were 39.98, 43.42 and 13.56 gL^{-1} in untreated corncob which was equal to the findings of Satimanont S. *et al*., (2012) with slight change in lignin quantity. Caustic and ammonium hydroxide removed about 84.0 and 70.0% of lignin at optimized condition. Sulphuric acid hydrolyzed more hemicellulose (93.0%) while phosphoric acid 89.0%. Silverstein *et al*., (2007) pretreated the cotton stalks with sulphuric acid, sodium hydroxide, ozone and hydrogen peroxide. Sulphuric acid hydrolyzed the hemicellulose 95.0% at 121°C and showed only 23.0% cellulose conversion yield during enzymatic hydrolysis. While sodium hydroxide and hydrogen peroxide credited about 65.0 and 29% delignification during pretreatment at 121°C.During acidic and alkali pretreatment amount of cellulose was increased due to reduction of lignin and hemicellulose content. Accellerase^{TM} 1500 along with Optimesh BG was used at specific concentrations to investigate the enzymatic hydrolysis performance of cellulose and hemicellulose present in the pretreated corncob. The lignocellulosic material converted into reducing sugars and then ferment into lactic acid by three-sequential steps, pretreatment, enzymatic hydrolysis and fermentation.

Effect of different catalysts on the biomass weight loss and hydrolysis: In order to obtain a specific comparison of different catalytic pretreatments on the biomass weight loss, removal of lignin, hydrolysis of hemicellulose, cellulose and amount of reducing sugars during pretreatment and enzymatic hydrolysis were determined by using different catalysts (H_2SO_4, HCl, H_3PO_4, $C_2H_2(COOH)_2$, CH_3COOH, NaOH, NH_3OH and H_2O) at different temperature for varying time. The reaction conditions for each catalyst, weight loss, amount of reducing sugars from pretreatment and enzymatic hydrolyzate for each condition was shown in Table 2.

Table 1. Chemical composition of corncob before and after pretreatment (most effective pretreatment)

Component	Before Treatment	H_2SO_4	Maleic acid	H_3PO_4	NaOH	NH_3OH
Extractives (wt% dry)	6.98±0.57	3.16±0.32	1.96±0.43	2.13±0.73	4.21±0.93	5.52±0.61
Hemicellulose (wt% dry)	43.42±1.45	2.64±1.02	5.54±0.96	4.82±0.82	14.53±0.51	17.71±1.25
Lignin (wt% dry)	13.56±0.74	6.34±0.59	8.56±0.88	7.54±1.32	2.08±0.39	3.93±1.43
Cellulose (wt% dry)	38.98±1.05	72.42±0.67	74.72±0.83	76.65±1.65	68.54±1.42	66.72±0.94

Table 2. The pretreatment conditions, amount of reducing sugars in pretreated and enzymatic hydrolyzate and hydrolysis yield in enzymatic media

Reagent	Temperature (°C)	Time for pre-treatment (h)	Decrease in biomass	Reducing sugars in hydrolyzate (gL^{-1})	Available biomass for hydrolysis	Enzymatic hydrolysis (% after 48h)	Reducing sugars after 48.0h (gL^{-1})
Sulphuric Acid	100	5	28.29	19.06±0.69	71.21±1.27	78.38±0.83	39.15
	110	5	21.67	15.27±1.34	78.33±0.92	79.96±2.39	39.35
	120	5	46.76	33.58±1.15	53.24±1.06	69.04±0.62	34.50
	130	5	58.16	38.39±0.73	41.84±1.59	73.33±2.02	36.65
	120	3	48.82	31.35±0.82	51.18±0.53	67.43±0.82	33.71
	130	0.5	49.40	30.43±0.43	51.60±0.27	72.84±1.30	36.38
Hydrochloric Acid	100	5	34.86	26.98±0.67	65.14±1.08	81.06±1.20	40.50
	110	5	31.92	21.05±1.8	68.07±1.70	79.19±1.21	39.55
	120	5	45.28	38.53±1.33	54.72±0.59	83.50±0.85	41.75
	130	5	58.45	41.34±0.70	41.55±0.57	85.06±1.44	42.50
	120	3	49.05	33.65±0.92	50.95±0.82	72.76±0.49	36.38
	130	0.5	50.32	31.75±0.43	49.68±0.35	73.43±0.62	36.57
Acetic acid	100	5	10.80	7.69±1.32	89.20±2.04	52.53±2.20	26.25
	110	5	13.57	9.49±0.75	86.43±1.55	59.66±1.09	29.80
	120	5	15.89	8.04±0.85	84.11±1.60	63.65±1.30	31.50
	130	5	29.70	14.38±1.79	70.30±1.05	64.98±0.99	32.45
	120	3	14.04	7.85±0.75	85.96±0.34	50.73±0.65	25.36
	130	0.5	24.93	11.72±0.20	75.07±1.03	53.78±0.52	26.85
Phosphoric Acid	100	5	28.26	20.17±1.06	71.74±1.68	89.04±1.02	44.50
	110	5	29.63	19.07±1.11	70.37±0.77	84.15±1.01	42.07
	120	5	43.54	30.69±1.01	56.42±1.11	83.66±0.40	41.80
	130	5	55.81	42.56±1.57	44.19±0.99	86.92±1.02	43.45
	120	3	37.85	27.09±1.2	62.15±0.12	76.14±0.72	38.07
	120	0.5	42.28	29.56±0.41	57.98±0.82	82.94±0.73	41.46
Maleic acid	100	5	28.93	20.97±1.43	71.07±1.69	81.56±1.05	40.75
	110	5	29.10	23.06±0.35	70.90±1.73	87.65±0.73	43.80
	120	5	40.60	31.31±0.88	59.40±1.14	83.83±1.36	41.90
	130	5	48.94	37.56±0.64	51.06±1.70	84.74±1.56	42.35
	120	3	36.98	28.65±0.78	63.02±0.51	76.50±0.61	38.25
	130	0.5	45.36	31.04±0.41	54.76±0.62	73.94±0.43	39.73
NaOH	100	5	13.53	NA	86.47±1.11	85.03±1.77	42.45
	110	5	14.56	NA	85.44±1.33	97.78±1.28	48.85
	120	5	64.37	NA	35.63±1.81	91.88±2.04	45.90
	130	5	61.71	NA	38.29±0.32	92.47±1.25	46.20
	120	3	27.87	NA	72.13±0.61	71.62±0.34	35.81
	130	0.5	41.05	NA	58.95±0.83	79.72±0.93	37.45
NH3OH	100	5	18.80	NA	81.20±2.33	80.76±1.59	40.35
	110	5	19.40	NA	80.60±0.59	90.66±0.42	45.30
	120	5	70.93	NA	29.07±1.37	96.42±0.99	48.20
	130	5	60.93	NA	39.07±1.37	92.07±1.77	46.03
	120	3	23.93	NA	76.07±0.82	74.32±0.62	37.16
	130	0.5	43.41	NA	56.59±0.73	72.03±0.61	34.01
Water	100	5	2.25	1.69±0.48	97.75±0.89	12.52±2.16	6.25
	110	5	2.53	1.78±0.52	97.47±0.68	13.76±1.05	6.85
	120	5	14.69	7.67±1.20	85.31±1.18	21.72±1.09	10.85
	130	5	16.78	10.42±0.92	83.22±1.91	19.42±1.29	9.70
	120	3	11.65	6.98±0.53	88.35±0.71	14.71±0.31	7.35
	130	0.5	10.34	5.43±0.42	89.60±0.54	12.54±0.93	6.27

The mineral acids showed 20.0-60.0% loss in weight of corncob at 100-130°C due to hydrolysis of hemicellulose in pretreatment step resulting in reducing sugars, while organic acids show 10.0- 15.0% less decrease in weight as compared to mineral acids and alkali. The sodium hydroxide and ammonium hydroxide showed 70.0% decrease in weight by removing the lignin and hemicellulose. The hydrolyzate obtained as a result of ammonium hydroxide and sodium hydroxide pretreatment showed no reducing sugars, while reduction in mass was occurred due to the hydrolysis of the hemicellulose and removal of lignin (Blasi D. *et al.,* 1999; Lin L. *et al.,* 2010; Li *et al.,* 2011). Alkali saponify the intermolecular ester bond present between the hemicellulose and other components which lead to hemicellulose separation from biomass along with disruption of lignin (Sun and Cheng 2002). It was observed that sodium hydroxide degraded the monomeric sugars while acids have no effect when pure glucose and xylose obtained at 100°C. Pure water showed only 15.0% of enzymatic hydrolysis yields when pretreated at 130°C for 5.0h. While sulphuric acid gave 79.9% hydrolysis yield when pretreated at 110°C which are close to 83.9% obtained by Ming *et al*., (2007) when pretreated at 108°C for 3.0h, hydrochloric acid showed 83.5% yield when pretreated at 120°C, phosphoric acid produce 89.0% when pretreated at 100°Cwhich was higher as compared to the results of Sartimanont S. *et al.,* (2012) and maleic acid gave 87.0% hydrolysis yield when pretreated at 110°C. Acetic acid produced only 64.0% conversion yield. The comparative hydrolysis efficiency was shown in fig.4. Enzymatic hydrolysis from sulphuric acid and hydrochloric acid provided almost pure glucose while alkali, provided a mixture of xylose and glucose (Fig.3 D).

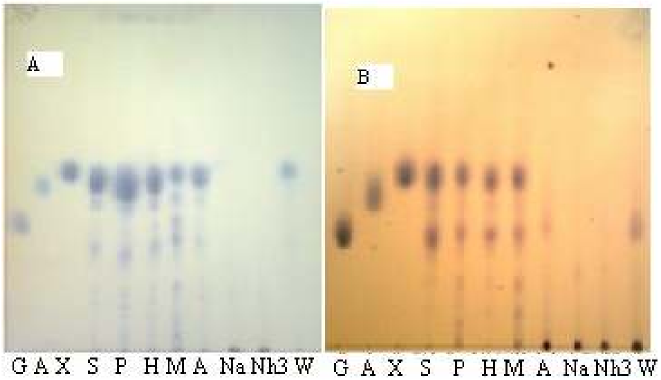

Figure 2. TLC images showing monomeric sugars in (A) pretreated at 130°C for 3 h,(B) at 130°C for 0.5h[G:glucose,A:arabinose,X:xylose,S:sulphuric acid, H:hydrochloric acid, P:phosphoric acid, M:maleic acid, A: acetic acid, NH_3:ammonium hydroxide, Na: NaOH, W:water]

***Effect of temperature on pretreatment*:** The effects of temperature (100, 110, 120, 130°C) on the pretreatment of corncob in the presence of different catalysts and subsequent enzymatic hydrolysis were studied. At low temperature, the solid residue left for hydrolysis showed higher hemicellulose content while at higher temperature maximum hemicellulose was depicted to be hydrolyzed into component sugars; glucose, arabinose and xylose, latter was dominant (Fig.2A), which results in decreasing the remaining polysaccharides or biomass as shown in Table 2. Higher enzymatic hydrolysis yield was obtained when corncob pretreated at temperature range 100-120°C with long incubation time. With the increase in temperature from 120°C to 130°C, the hemicellulose was completely removed when pretreatment was done with acids, as there was no xylose in TLC images of enzymatic hydrolyzate obtained at 130°C (Fig.3D).

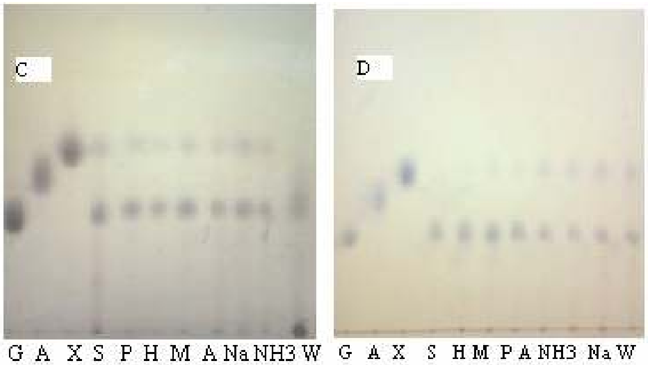

Figure 3. TLC images showing monomeric sugars after enzymatic hydrolysis (C) pretreated at 120°C for 3 h, (D) at 130°C for 0.5h. [G: glucose, A: arabinose, X: xylose, S: sulphuric acid, H: hydrochloric acid, P: phosphoric acid, M: maleic acid, A: acetic acid, NH_3: ammonium hydroxide, Na: NaOH, W: water]

H_2SO_4, Maleic acid and NaOH pretreatment provided 39.12±0.5, 43.54±0.5 and 48.34±1 gL^{-1} of reducing sugars during enzymatic hydrolysis at 110°C respectively, while HCl and NH_3OH provided 40.0±0.5 and 47.0±1.0 gL^{-1} at 120.0°C, H_3PO_4 gives 44.0 gL^{-1} at 100.0°C.With the increase in temperature, the reducing sugars produced from hemicellulose were converted into furfural as spots are visible at upper edge of TLC images of hydrolyzate obtained at 130°C (Fig. 2A) which was also mentioned by different authors (Ackerson *et al.,* 1981: Taherzadeh *et al.,* 2007). The production of furfural depends on the acid concentration and temperature (Gonzales *et al.,* 1986). To overcome degradation of hemicellulose sugars during pretreatment temperature should be kept at low for maximum recovery of xylose/glucose. Saha B.C. *et al*., (2005) described that at temperature 121°C, hemicellulose hydrolyzed into component sugars (xylose, arabinose & galactose) without any further degradation into furfural and hydroxymethyl furfural. The hydrolysis efficiency of acid pretreated corncob was higher at lower temperature array (100-110°C), also mentioned by Pattana L. *et al.*, (2009).

Effect of residence time during pretreatment step**:** The cellulosic residue and the byproducts formed during pretreatment step were different for varying time at different temperatures. The amount of reducing sugars was less at short time with the production of furfural and 5-hydroxy furfural which results from the glucose and xylose degradation. It was cleared that at high temperature (150-190°C) and short time of pretreatment the amount of furfural and 5-hydroxyfurfural was greater as determined by Girisuta B. *et al.,* (2008). The amount of reducing sugars at high temperature and time were found to be higher during pretreatment steps. The reducing sugars in enzymatic hydrolyzate were higher for long time of pretreatment at lower temperature while at higher temperature; short time of pretreatment was more effective (Satimanont S. *et al.,* 2012; Pattana L.*et al.,* 2009).When corncob was pretreated for 3.0h with 1.0% sulphuric acid and sodium hydroxide at 120°C, 57.0 and 72.0% hydrolysis yield was obtained which increases to 78.0 and 96.0%respectivelywhen residence time was increased at 5.0h.The time and temperature during pretreatment significantly ($p<0.0001$ with R^2: 0.9862) affect the amount of reducing sugars and hydrolysis yield.

Most effective pretreatment conditions**:** Corncob contains cellulose as major component (35-55%), along with hemicellulose (25-35%), lignin and some other materials (Sun and Cheng 2002) which were removed during pretreatment steps. The pretreatment of corncob, either with acids or alkali results in loss of biomass weight by removing the lignin (Blasi *et al.,* 1999; Lin L. *et al.,* 2010) and hydrolysis of hemicellulose (Jiele *et al.,* 2010; Ziyu *et al.,* 2010). At low temperature this loss is less and at higher temperature more mass lost. Mineral acids lost more (<50.0%) as compared to maleic acid (organic acid: below 50.0%) while alkali showed more than 60.0% loss in biomass. When pretreatment was done with NaOH, lignin content swells (Carillo *et al.*, 2005) and alters the morphology and crystallinity of the cellulose which increases the cellulose digestibility during enzymatic hydrolysis (Ai-zuhair 2008). The NaOH pretreatment provided higher enzymatic saccharification as compared to acids (Zhao *et al.,* 2008; Aswathy U.S. *et al.,* 2010). Acids provided reducing sugars for fermentation during pretreatment step while alkali degrades them. Approximately 40$\underline{+}$2% reducing sugars were available for fermentation (Jing-Ping *et al.,* 2011; Masami G.O.O. *et al.,* 2008) during pretreatment step at 110 -130°C (Fig. 4), which contains xylose as the major component. All acidic pretreatment showed enzymatic hydrolysis yield greater than 80.0% and alkali 95.0% which was higher as compared to acids. The loss of biomass, production of reducing sugars during pretreatment and the yield of enzymatic hydrolysis showed that acid pretreatment especially phosphoric acid and maleic acid at moderate temperature(110-130°C) was favorable catalyst used for pretreatment and production of fermentable sugars through enzymatic hydrolysis from corncob waste (Fig.4).

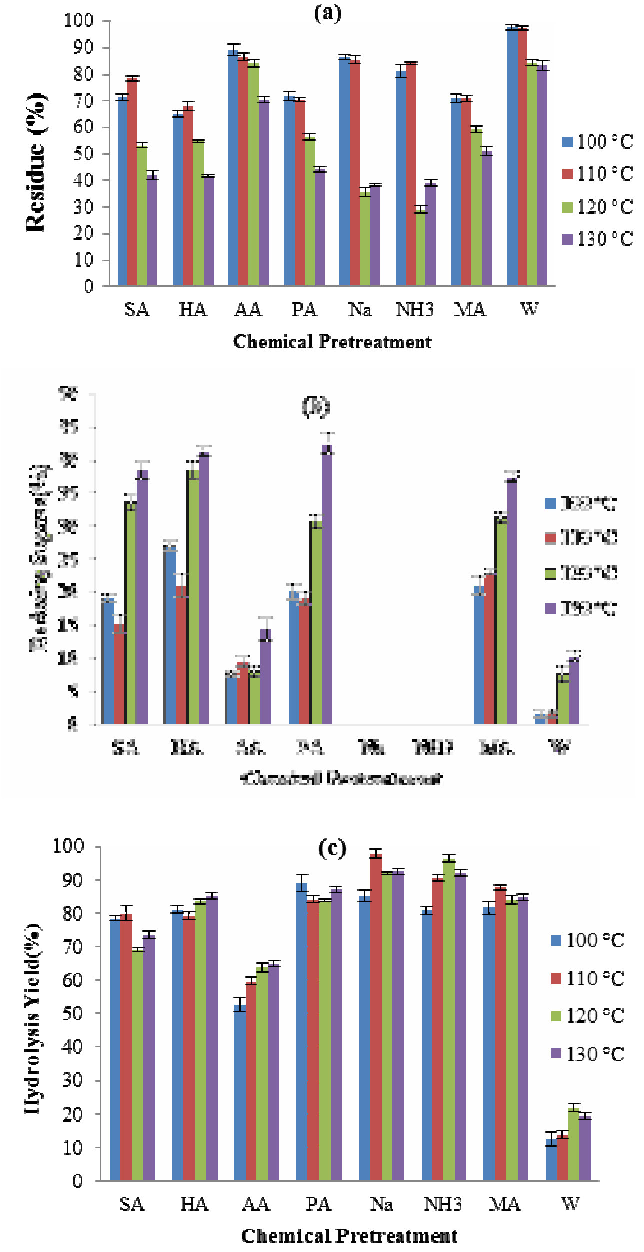

Figure 4. (a) Effect on biomass weight; (b) amount of reducing sugars (c) enzymatic hydrolysis yield (SA: Sulphurc acid, HA: Hydrochloric acid, AA: Acetic acid, PA: Phosphoric acid, MA: Maleic acid, W: water)

Lactic acid production from enzymatic hydrolyzate: Sugars present in enzymatic hydrolyzate of corncob were used for the production of lactic acid. The reducing sugar concentration was adjusted at 40.0 gL^{-1} by diluting the hydrolyzate except acetic acid which was at 30.0 gL^{-1}. Pure glucose and enzymatic hydrolyzate obtained from acid treated corncob was greater than 90.0%. John *et al.,* (2011) obtained 97.3% conversion yield of lactic acid using *lactobacillus pentosus* with 0.83 gg^{-1} of sugar. Phosphoric and maleic acid treated corncob produced 30.86 and 31.55 gL^{-1} of lactic acid with 0.92 & .93 gg^{-1} of yield. Wang L. *et al*., (2010) obtained 74.7 gL^{-1} of lactic acid from corncob with 0.50 gL^{-1}h^{-1} productivity. Hydrochloric and sulphuric acid treated corncob hydrolyzate showed 29.86 and 30.37 gL^{-1} of lactic acid along with 0.74 and 0.73 gg^{-1} of yield which was greater than the quantity (17.3gL^{-1}) obtained by Ali Z. *et al.,* (2009). Miura *et al.,* (2004) obtained 25.0 gL^{-1} of lactic acid by using Rhizopus MK-96-1196 strain from sulphuric acid treated corncob hydrolyzate which has 55.0gL^{-1} of sugars. All acidic hydrolysate have productivity in the range of 0.74-0.78 gL^{-1}h^{-1} (Table 3). Lactic acid from acetic acid treated corncob hydrolysate was less in quantity having productivity 0.44 gL^{-1}h^{-1}. Sugar analysis of fermenting media showed that acid treated hydrolyzate after lactic acid fermentation has less reducing sugars while alkali treated hydrolyzate showed more sugars (Fig. 5). Only xylose was present in images of tlc obtained after fermentation which is less consumed by the lactobacillus (Fig.6). Alkali treated hydrolyzate produced less lactic acid yield as compared to acid treated materials. Hydrolyzates obtained from sodium hydroxide and ammonium hydroxide produced 24.86 and 24.11gL^{-1} of lactic acid with 0.6 and 0.62gL^{-1}h^{-1} of productivity.

Conclusions: Corncob could be hydrolyzed by chemo-enzymatically in two steps. Alkali pretreatment was efficient as compared to acids, to enhance the enzymatic digestibility of corncob with the removal of more lignin. Reducing sugar yield produced by enzymatic hydrolysis of NaOH, NH_3OH, H_3PO_4 and $C_2H_2(COOH)_2$ treated corncob were 48.5±0.6, 47.3±0.5, 44.0±0.5 and 43.6±0.5 gL^{-1} respectively. Acetic acid and pure water treatment provide less reducing sugars, 30.0±1 and 10.0±1.0 gL^{-1}respectively. The availability of 40.0±2 gL^{-1}reducing sugars during acidic pretreatment step makes it more lucrative as compared to alkali. Reducing sugars obtained from enzymatic hydrolyzate were successfully converted into lactic acid with 90.0% conversion yield. Keeping in view the pretreatment step with acids at temperatures 100-130°C, time 0.5-5.0h, acid catalyzed enzymatic hydrolysis of corncob could be a low cost process for the production of fermentable sugars at industrial level which ultimately converted into lactic acid and other valuable chemicals like acetic acid, formic acid, citric acid and ethanol.

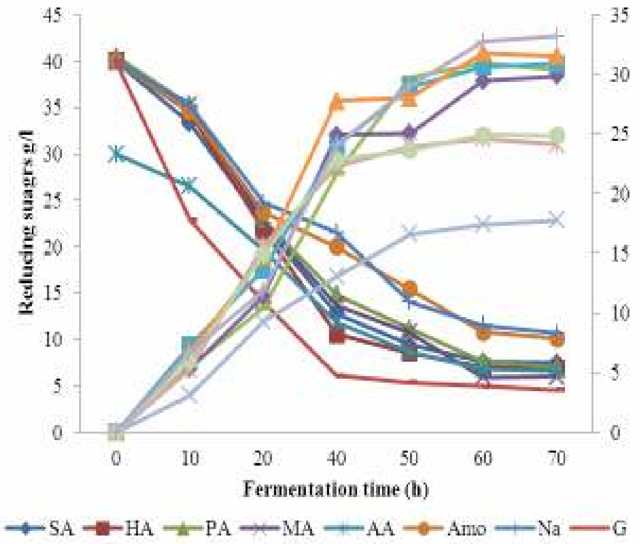

Figure 5. Production of lactic acid and consumption of sugars during batch culturing (SA: sulphuric acid, HA: hydrochloric acid, PA: phosphoric acid, MA: maleic acid, AA: acetic acid, Amo: ammonium hydroxide, Na: NaOH, G: glucose)

Table: 3 Kinetic parameters of lactic acid fermentations by batch culturing of *lactobacillus acidophilus*

Pretreatment Reagent (1.0%)	γ(Initial Sugar Conc.) gL^{-1}	γ(Lactic acid) gL^{-1}	η(Lactic acid) gg^{-1}	Productivity gL^{-1}h^{-1}
Sulphuric acid	40.0	30.37±0.32	0.90	0.75
Hydrochloric acid	40.0	29.86±0.73	0.90	0.74
Phosphoric acid	40.0	30.86±0.40	0.93	0.77
Maleic Acid	40.0	31.55±0.67	0.92	0.78
acetic acid	30.0	17.77±0.70	0.75	0.44
Ammonia	40.0	24.11±0.83	0.83	0.60
Sodium Hydroxide	40.0	24.86±0.48	0.84	0.62
Glucose	40.0	33.18±0.91	0.93	0.82

η (lactic acid) =Lactic acid produced/sugar consumed (g/g)

Figure 6. TLC images showing monomeric sugars after lactic acid production [G: glucose, A: arabinose, X:xylose, S: sulphuric acid, H: hydrochloric acid, P: phosphoric acid, M: maleic acid, A: acetic acid, NH_3:ammonium hydroxide, Na: NaOH, W:water]

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