The enzymatic hydrolysis and fermentation of pretreated wheat straw and bagasse to ethanol

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Abstract

Saline sodic soils have been used for production of biomass using salt tolerant perennial grasses, namely *Leptochloa fusca* (kallar grass) followed by lesser tolerant plant, namely, Panicum maximum to provide biomass throughout the year for production of single cell protein, celllases hemicellulases and ethanol. The concentrated enzyme titers obtained after growth of C. biazotea on kallar grass using sodium nitrate as nitrogen source could saccharify alkali-treated wheat straw, bagasse, carboxy methyl cellulose and alpha-cellulose to monomeric sugars (› 88% theoretical yield) but not kallar grass straw. In simultaneous saccharification and fermentation studies, 94 and 90 % theoretical yield of ethanol from wheat straw and bagasse from 10% carbohydrates in both substrates were achieved compared with alpha-cellulose, which supported 96 % theoretical yield of ethanol.

1.Introduction

Photosynthesis is the most efficient method for harnessing solar energy for biomas production. The global production of lignocellulosic (LC) material from the land is about 120-150 billion tons dry matter/annum, some 302 billion ton oil equivalent (TOE) or more than four times the world's yearly total energy consumption [1]. In Pakistan, LC materials are the most important components of the renewable biomass and contributed 17.7 million ton oil equivalent (MTOE) per annum fuel wood, animal waste, cotton sticks and other crop residues in year 2002-2003 against anticipated demand of 83.7 MTOE. During the recent decades, Pakistan has experienced fairly good economic growth but the country is still at a low level of economic development and in coming decades, will need large inputs of energy in order to sustain the pace of its economic development. Presently, 68% of primary energy needs of the country are met by commercial fuels (oil, natural gas, coal, hydro- and nuclear- power) and 32% by traditional fuels (fuel wood, crop residues and animal wastes). Since Pakistan is a resource deficient country so it has to rely on imports to cover about one third of her primary energy demands [1].

Vast areas of salt affected wasteland available in Pakistan can be utilized for the production of energy crops by growing the salt tolerant plants for the economic production of fuel and other products. Because of its C-4 system of photosynthesis, a high tolerance to salinity and sodicity, and associative nitrogen fixation, *Leptochloa fusca* L. kunth (kallar grass) is able to produce (with a production cost of $3.0/metric ton) 50 ton biomass/ha and could be a cheap source of capturing solar energy in developing countries [2].

Grasses, trimmings of lawns, other agriculture wastes, industrial, domestic, food and urban solid wastes are currently over produced (over 43 million tons per year, 2) but under utilized. Recycling these wastes would not only aid in pollution abatement but can also serve as a vital source of energy and food for the future. The use of wastes for methane or ethanol production will reduce overall cost and make the process economically viable.

The removal of lead or toxic aromatics used in gasoline as octane boosters has provided new application and markets for alcohol to enhance octane rating and provide an alternative liquid fuel. Ethyl alcohol is gaining increased acceptability as fuel as it burns clean and can be quite easily blended with gasoline. Molasses has been used in our laboratories to produce ethanol using *Saccharomyces cerevisiae* at bench scale, as well as in continuous culture [1] at industrial scale. But the cost of molasses is increasing rapidly and distillers are concerned by the price hike. Other attractive alternative is the readily available lignocellulosic (LC) biomass, which has a considerable promise as a raw material for liquid fuels, and certain petrochemical intermediates, as this is renewable.

Utility of cellulose in LC biomass is tremendously enhanced if it is first hydrolyzed to glucose and other soluble sugars that in turn can be used for making sweeteners, single cell protein (SCP), energy materials (alcohol) or other fermentation products. For production of ethanol from LC biomass, one of the designed processes is enzyme/acid hydrolysis followed by fermentation to ethanol. Acid hydrolysis has been commercialized but it has drawbacks of low yield of ethanol, extensive corrosion problem or energy intensive if acid hydrolysis is carried out with diluted acids at 180°C. Increasing knowledge of enzymes and their mode of action as well as their recent applications have greatly expanded the prospect for enzymatic process [3]. The major attribute of the enzymatic approach is its potentially high saccharification efficiency observed by many workers [4,5]. For saccharification of pretreated substrates, co-operative action of a complex of exo-glucanase or cellobiohydrolases (EC 3.2.1.91), endo-glucanase (EC 3.2.1.4) and ß-glucosidase (EC 3.2.1.21) is involved in breakdown of complex carbohydrates present in LC biomass (Rajoka *et al.* 1998), the end product is glucose which can be sequentially fermented to ethanol. *Cellulomonas* spp. have been widely used for production of cellulases from LC biomass [6] and their enzymes have been extensively used in saccharification and ethanol production studies from agricultural residues [7]. In this study concentrated cellulases and hemicellulases were used to saccharify LC and cellulosic substrates. Wheat

straw and bagasse, which were easily saccharified, were converted into ethanol using simultaneous saccharification and fermentation to ethanol. Information has been added on the kinetics of its production from LC biomass.

2. Materials and Methods

2.1 Materails. α-Cellulose, cellobiose, Sigmacell 100, Larchwood xylan, *p*-nitophenyl-ß-D Glucopyranoside (pNPG), *p*-nitrophenyl-ß-D-xylopyranoside (pNPX), *p*-nitrophenyl_ß-D-cellopyranoside (pNPC), carboxy methyl cellulose, Na-salt (CMC, low viscosity with degree of substitution 0.78), were purchased from M/S Sigma Chemical Company, St. Louis, MO 63178 USA. All other chemicals were of analytical grade. Kallar grass straw, and wheat straw were obtained from Biosaline Research Substation (BSRS), near Lahore. Bagasse was a gift from Crescent sugars mills, Faisalabad.

2.2 Organism. Strain of *Cellulomonas biazotea* NIAB 442, collected from Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan, was maintained on Dubos salt-α cellulose solid medium containing (g/l) K_2HPO_4 0.5 g, $NaNO_3$ 4 g, KCl 1.0 g, $MgSO_4$ $7H_2O$ 0.5g, $FeSO_4.5H_2O$ 0.0 1 g, α -cellulose 10 g, yeast extract 2 g (pH 7.3), and agar 20 g. *Cellulomonas biazotea* was cultured in Dubos salt-cellobiose yeast extract medium overnight at 30°C in Erlenmeyer flasks as described previously [6] and used at 10% (v/v) basis to inoculate the growth medium. A thermotolerant mutant of *Saccharomyces cerevisiae* ATCC 26602 [8] was used as ethanologenic organism. It was maintained on the medium described previously [3]. The strains were cultured routinely every fortnight. For preparation of inoculum, a loopful of culture from slant was transferred to the inoculum medium containing basal salts and 1% (w/v) glucose at 30 °C.

2.3 Preparations of Lignocellulosic (LC) substrates: Lignocellulosic (LC) substrates were air dried after rinsing with tap water. The dried biomass was made into a 55-mesh powder by grinding in an electric grinder. The powdered biomass was given alkali pretreatment separately with 10% NaOH keeping biomass alkaline solution ratio at 1:10. The treated substrates were autoclaved for 15 min, washed to neutralize and again dried in the oven at 80 °C. The dried biomass had 10% moisture content. The LC biomass was analyzed for car-bohydrate content as described previously [4]. Kallar grass straw, wheat straw and bagasse contained 78.0±6.0, 88±7.5 and 84 ± 7.6 % total carbohydrates

2.4 Growth studies with *C. biazotea*: Enzyme production in all strains of *Cellulomonas* spp. from CMC, α-cellulose or Sigma cell 100 (0.25-1.5%) indicated that 1% (w/v) concentration was (in liquid medium) optimum for biosynthesis of all enzyme activities observed [7]. Enzymes were produced with 1% cellulose + hemicellulose in kallar grass biomass in 1 litre Erlenmeyer flasks with 200 ml of culture medium. Flasks were inoculated with 20 ml overnight culture of test organism with OD of 10 x 0.3, prepared as above. The culture flasks were incubated at 30 °C on a gyratory shaker (100-revolution min^{-1} for 72 h. The unused substrate was removed by filtra-tion through Cheesecloth.

Cells were harvested by centrifugation (4°C, 15 min, 12000 g), and the supernatant used as extra cellular fraction. Cells were suspended in chilled McIlvain's phosphate buffer (pH 7), and disrupted by probe sonication in two bursts of 1 min with Braun sonicator 2000 at low density with at least 60-second intervals between disintegrations. The sonication vessel was cooled in crushed ice throughout. Supernatant from this disruption was used as the intracellular enzyme preparation. In some experiments, the pelleted samples were resuspended in a minimum volume of buffer and dialyzed at 4°C overnight to remove sugars remaining from the growth medium and soluble products of cellulolytic action formed during growth, both of which might interfere with the product. The enzyme activities in the extra cellular (soluble), and intracellular were assayed for cellulase and xylanase enzymes.

2.5 Enzyme assays: Endo-glucanase (CMC-ase), exo-glucanase or cellobiohydrolase activities were determined as described previously [6,9,10]. For endo-glucanase, the total reaction mixture of 3 ml contained 1 ml of carboxymethyl cellulose Na salt (CMC, 1% w/v) 1 ml phosphate citrate buffer (50 mM pH 7) and 1 ml of appropriately diluted enzyme. After incubation for 30 min at 40 °C, the reducing sugars were estimated as glucose equivalent by the dinitrosalicyclic acid (DNS) method [11].

Filter paper (FP-ase) activity was determined under similar conditions as described above except that 6x1 cm What man no. 1 filter paper was used as a substrate in place of CMC. Xylanase activity was also determined under similar conditions as above except finely powdered Larchwood xylan (Sigma) suspension was used as a substrate. The reducing sugars were measured as xylose equivalents by the DNS method [2]. The units are international units (µM reducing sugars equivalent glucose or xylose/ml or specific activity was determined.

ß-Glucosidase, ß-xylosidase or cellobiohydrolase was assayed according to method described previously [2, 6] with some modifications. The total assay mixture of 3 ml consisted of 1 ml of the substrate (5 mM pNPG, pNPC or pNBG)), 1.0 ml buffer and 1.0 ml of suitably diluted enzyme. The reaction was initiated by addition of activated enzyme (enzyme incubated for 10 min at assay temperature and called activated enzyme) followed by incubation at 40°C for 10 min. The reaction was terminated by addition of 3 ml of sodium carbonate (2%) and the para-nitrophenol liberated was determined as absorbance at 410 nm. Cellobiase was determined from glucose released from 1 mM cellobiose by glucose oxidase method. One unit of activity defined as micromoles of product (para -nitrophenol or glucose) formed per minute per ml of enzyme preparation. Protein was estimated according to method of Lowry et al. [12] with bovine serum albumin.

2.6 Saccharification studies: Portions of 0.2, 0.375, 0.5 or 0.625 g of bagasse, kallar grass straw, wheat straw or CMC were dispensed into 50 ml Erlenmeyer flasks. One ml phosphate citrate buffer (pH 7) containing 0.02 ml of tween 80 was added to each flask. They were incubated at 40°C in shaking water bath incubator along with 4 ml of concentrated enzyme preparation whose pH was brought to 7 with 1 N HCl. The crude enzyme was obtained from C. biazotea grown on 1.25% kallar grass straw liquid culture and concentrated. For the latter, the extra cellular and intracellular preparations (the later prepared in one tenth of original volume) were mixed, dialyzed and concentrated by ultra filtration (with Amicon concentrator fitted with cutoff size membrane of 10 kDa) to contain 10 mg/ml protein and assayed to possess desired enzyme activities. Duplicate flasks were harvested periodically and properly diluted aliquots were used to determine reducing sugars measured as glucose using dinitrosalicylic acid method..

2.7. Ethanol production studies: For ethanol production studies (a) five, and 7.5 g of alkali treated wheat straw, or bagasse, 0.5 g of yeast extract and 0.25 ml of Tween 80 were added to a 250 ml Erlenmeyer flasks separately with 10 ml of buffer of pH 5.0 and salts [(K_2HPO_4 10 g, $MgSO_4$ $7H_2O$ 5 g, $(NH_4)_2SO_4$ 5g and yeast extract 2 g (pH 5.0)], medium and autoclaved. Eighty ml of a predetermined cellulase preparation (pH 5) were added and incubated in a reciprocal shaker at 100 revolutions per minute for 4 h. Ten ml of an overnight culture of *S. cerevisiae* mutant was added and the flasks were re-inucbated for various times. b) Ten and 12.5 g of wheat straw, and bagasse, 2.5 g yeast extract and 0.25 ml of tween 80 were added to 250 ml Erlenmeyer flasks containing 10 ml of buffer of pH 5.0 and salts as above. The flasks were autoclaved and 80 ml of concentrated enzyme preparation were added to produce a working volume of 90 ml. After incubation for 4 h, 10 ml culture of *S. cerevisiae* mutant was added and the flasks were re-incubated for specified times . The experiments were repeated in (a) and (b) but with same concentration of α-cellulose (c).

Duplicate flasks were harvested. The insoluble substrate was centrifuged and supernatant was assayed calorimetrically to assay for sugars using dinitrosalicylic acid, glucose was measured using glucose oxidase kit, cell mass determined gravimetrically and ethanol was determined using Perkin Elmer gas chromatograph equipped with FID detector using chromos orb 101 column at 151°C. N_2 was used as a carrier gas while injector and detector was 200 and 220 °C respectively. Concentrations were calculated using ethanol as standard.

3. Results and discussion

The enzymes were prepared after growth of C. biazotea on 1% (w/v) carbohydrates in alkali treated kallar grass in Dubos-slats medium containing 0.4% sodium nitrate (pH 7.3) for 72 h at 30°C. The extra cellular and cellular fractions prepared as described in Materials and Methods were mixed and concentrated to contain 32.0, 10.0, 11.0, 10.0, 200.0, 11, and 25 IU/ml for endoglucanase, exo-glucanase (FPase), ß-cellobiohydrolase,, ß-glucosidase, xylanase, cellobiase and ß-xylosidase respectively (Table 1). Representative figure for saccharification of 5 (Fig. 1a) and 12.5% (Fig1 b) CMC, α-cellulose, kallar grass, bagasse and wheat straw is presented (Fig.1). These enzyme titers showed high saccharification of wheat straw, bagasse and α--cellulose (>90%) and markedly enhanced carbohydrate productivities (Table 2). In saccharification studies, when the reaction mixture contained above units of CMC-ase, FPase, ß-glucosidase, cellobiase and ß-cellobiosidase/ml, a high saccharification was an expected proposition [13]. The concentration of FPase in the reaction mixture (containing 12.5% substrate suspension) was 64 IU/g substrate. Normally 15-40 units/g substrate have been found to cause 80-90% saccharification [13]. Since the enzyme concentration was up to 3- times higher than the recommended dose, a high saccharification rate was expected [4,7]. This finding is consistent with finding on saccharification values obtained with other cellulases [3, 14, 15]. At high substrate concentration, low saccharification rate has been reported even when 40 IU FPase/g substrate, was used [13]

In ethanol production studies, the saccharification was carried out for 4 h and then fermentation was brought about by adding an inoculum of *S. cerevisiae*. The contents were incubated at 40°C with shaking at 100 rpm (Fig. 2). At predetermined intervals of time, duplicate samples were harvested and assayed for ethanol (p), reducing sugars (s) and cell mass (Fig.2) from 12.5% concentration of wheat straw (a), bagasse (b), and α--cellulose (c). *S. cerevisiae* utilized the sugar produced from the substrates present in the fermentation vessel, for synthesis of cell mass and ethanol. After 25 h, the culture reached the stationary growth phase and fermentation was terminated.

The final concentration of FPase and ß-glucosidase was 64 and 57.6 IU/g substrate each compared against (ca) 30 IU/g substrate found optimum for conversion to glucose [4]. The maximum yield of ethanol was 5.3, 4.8 and 6.12 from wheat straw, bagasse and α-cellulose respectively ca 6.4 % produced from glucose. These values are markedly higher than those reported by previous authors [3, 14-16].

An increase in the amount of straw in the reaction medium from 5% to higher concentration limited the degree of saccharification and glucose yield in the saccharified syrup. This was due to difficulties which were encountered in stirring the biomass, thereby decreasing the ethanol production rate [13]. In the present study, this trend was not visible, as the hydrolytic products did not significantly inhibit the growth of the yeast as was reflected in high cell mass yield (9.0 g/litre) comparable with that formed on glucose and that reported by Szczodrak [13] while fermenting 10% wheat straw whereas ethanol yield is comparable with that reported by Szczodrak [13]. In the present studies, the cellulase used had optimum enzyme activity at 40°C and *S. cerevisiae* mutant shows $Y_{p/s}$ comparable to one at 30°C, better performance as far as ethanol production is concerned was reached.

Table 1. Enzyme activities of *C. biazotea* cellulase preparations made up to protein concentration of 5 mg/ml compared with the activity of enzyme preparation with 1 mg/ml protein content. Activities have been expressed in IU/ml

Enzyme	Fermentation broth (1.0 mg/ml)	Concentrated (5 mg/ml)
Endoglucanase	7.0	32.0
FPase	2.4	10.0
Xylanase	50.0	200.0
ß-Gucosidase	2.1	9.0
Callobiase	2.4	10.0
ß-Xylosidase	6.0	25.0
ß-Cellobiosidse	2.8	11.0

Table 2. Productivity of reducing sugars (Q_{RS}, g per l per h) from 5.0 , 10.0 and 12.5 % CMC, a-cellulose, wheat straw, kallar grass and bagasse using concentrated cellulase and hemicellulase preparations

Substrate	Q_{RS} (g per l per h) from substrate concentrations		
	5%	10%	12.5%
CMC	5.6	13.0	16.0
α-Cellulose	4.9	11.8	12.5
Kallar grass	2.6	6.2	6.4
Wheat straw	3.2	7.5	8.5
Bagasse	2.7	6. 7	7.9

Table 3. Comparative kinetic parameters for production of ethanol from 5 and 7.5.5% wheat straw, bagasse and α-cellulose using cellulases[a] from *C. biazotea* and cultures of *S. cerevisiae* thermotolerant mutant

Kinetic Pa-	Wheat straw	Bagasse	α-cellulose	Wheat straw	Bagasse	α-cellulose
	-------------------5%---------------			---------------7.5%-----------		
μ (h^{-1})	0.21	0.19	0..23	0.24	0.23	0.26
$Y_{p/s}$ (g/g)	0.49	0.48	0.50	0.48	0.47	0.50
Y_t (mg/ml)	53.00	24.00	25.50	37.00	36.00	38.00
Y_t	85.00	80.00	96.50	67.30	60.00	100

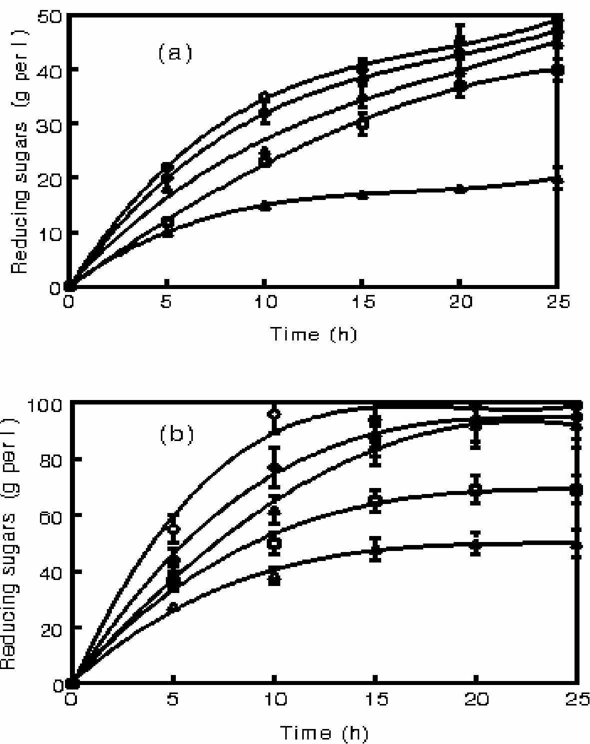
C. biazotea was grown on 1.25% alkali treated kaller grass straw [7] to prepare enzyme filtrates which were concentrated and assayed to contain desired enzyme activities (Table 1) and * μ (h^{-1}) growth rate; $Y_{p/s}$ ethanol yield; Y_t, theoretical yield (calculated on the basis of total cellulose and hemicellulose content); % Y_t, percent theoretical yield, were determined as described previously [7,8]. Wheat straw and bagasse had 88 $\pm$ 1.0 and 84- $\pm$ 1.5%

The cost of production of ethanol from molasses in Rs. $0.31/litre (96.4% ethanol) from a 200 m^3 fermentor, its cost from LC biomass is calculated to be $0.42/litre because of enzyme production and substrate preparation costs. The gate price will be reduced if spent-lignin is used for energy generation and SCP (which has all amino acids required for experimental animals), is sold as poultry feed supplement, then production price of ethanol from LC biomass will be comparable to that from molasses.

The liquid fuel production technologies may yield 2.0 to 2.5 ton oil/ha by growing oil yielding plant on fertile lands while ethanol production can reach 2.0-2.3 ton/ ha from kallar grass grown on saline lands. Though energy efficiency of ethanol producing system is smaller. For instance, energy input for producing biomass is 12 MJ/kg of ethanol produced. The energy input or processing technologies is 12MJ/kg of ethanol compared as energy content of ethanol 28-29 MJ/kg of ethanol + 3.5-3.8 MJ/kg ethanol by product energy produced in the form of single cell protein +17 MJ/kg of spent waste energy produced from lignin or extraction of chemicals [1].

For reducing the cost of saccharification, we isolated genes coding for cellulases from C. biazotea and first cloned in E. coli and subsequently in yeast, Saccharomy-

Figure 1 Kinetics of production of reducing sugars from CMC (O), α-cellulose (●) kallar grass straw (Δ), wheat straw (▲) and bagasse (ĺ) during saccharification process respectively. Cellulases and xylanases were produced using 1.25 % kallar grass in shake flask cultures using optimized Dubos salt medium [7]. Clear supernatant was obtained by centrifugation and was concentrated using Amicon concentrator fitted with 10 k Da cut-off size membrane. The saccharification was brought out with concentrated enzyme preparation containing 64 and 57.6 IU of FPase and ß-glucosidase/g substrate respectively. Error bars show standard deviation between duplicate readings.



ces cerevisiae to see their expression with an express aim of introducing cellulolytic character in yeast for large scale production of enzymes with concomitant production of ethanol in a single step and development of new processes using plant raw materials or their products. The yeast harboring genes secreted the gene products in the culture medium, and performed post-transnational modification. We intend to improve this trait by cloning the cellulase genes under the influence of strong promoters to achieve hyper production of these enzymes for commercial exploitation. Our ultimate objective is to chromosomally integrate cellulase genes [17] under the influence of a strong promoter and also transfer plasmid borne genes coding for industrially important proteins for their co-production from inexpensive agro-industrial wastes. Fermentative ethanol production from kallar grass can give 4.5 X 10^8 gallon per year. Micro-organisms such as genetically *S. cerevisiae* could be tailored to produce variety of enzymes as co products with ethanol; 5% of cell protein would produce up to 145, 000 kg of enzymes as co products. These enzymes cold include enzymes for large markets such as detergent industry, the textile industry, the food industry, and the wood pulp industry and biocatalyst-based industries for novel chemical; with the availability of new markets, the value of these enzymes could exceed the current value of ethanol itself.

Conclusions

Concentrated enzyme syrup saccharified CMC, alpha-cellulose, wheat straw, bagasse and kallar grass to reducing sugars with high saccharification rates. In combined saccharification and fermentation, alpha-cellulose, wheat straw and bagasse hydrolysates were converted into ethanol with 94, 90 and 86% theoretical yield of alcohol respectively. Energy from biomass is an important way to improve security in energy supply for the future. The bioenergy sources are decentralized, cheap, renewable and suitable for continuous energy supply and for their conversion into various more valuable secondary energy carries.

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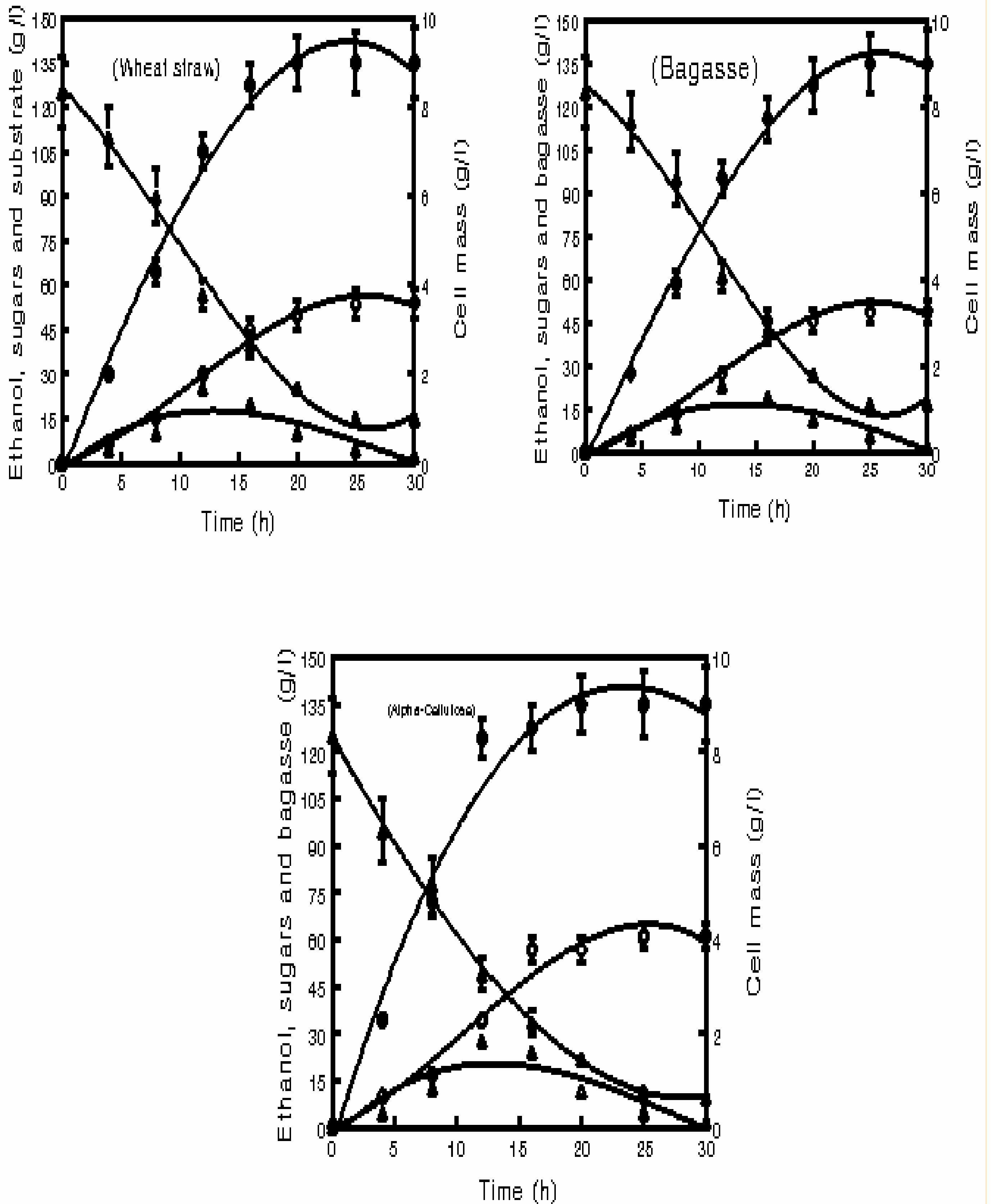
Figure 2. Kinetics of ethanol (O), cell mass (●) production, accumulation of total reducing sugars (Δ) and solid substrate (▲) in the fermentation broth during combined saccharification and fermentation process respectively. Cellulases and xylanases were produced as above in shake flask cultures. The saccharification was brought out for 4 h and the syrup was inoculated with yeast mutant culture. Error bars show standard deviation between duplicate readings.



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