BIOETHANOL PRODUCTION FROM THE PODS OF *Samanea saman* (Jacq.) Merr.

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BIOETHANOL PRODUCTION FROM THE PODS OF *Samanea saman* (Jacq.) Merr.

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CERTIFICATE

This is to certify that the thesis entitled "**BIOETHANOL PRODUCTION FROM THE PODS OF *Samanea saman* (Jacq.) Merr.**" *submitted by* **Miss. Vidyavathi, K.B.**, for the degree of **MASTER OF SCIENCE (AGRICULTURE)** *in* **AGRICULTURAL MICROBIOLOGY** *to the University of Agricultural Sciences, Dharwad, is a record of research carried out by her during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.*

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DHARWAD
FEBRUARY, 2003
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INTRODUCTION

I. INTRODUCTION

Energy requirement and environmental pollution are the two major challenges today. Energy has been always in demand not only in the past but is a continuing crisis due to the advance technologies, and increase in population. The fuel crisis has been precipitated because of fast depletion of the naturally occurring conventional fossil fuel reserves such as petrol, diesel, kerosene, coal, etc (Ramanathan, 2000). It is the transport sector that consumes majority of the petrol and diesel. The number of vehicles registered in 1951 were 3 lakhs, which has increased to 531 lakhs in 2000-01 (Uppal, 2001) indicating the constant demand of fuel. Our sole dependence on petrol or diesel not only increases financial burden but also leads to environmental pollution due to the emission of smoke containing lead, benzene, sulphur dioxide, nitrogen oxide, carbon monoxide etc. These gases contribute to 64 per cent of air pollution in our metros and adjoining suburbs, there by, leading to health hazards *viz.*, cancer, bronchial infections, pneumonia, etc. (Das *et al.*, 2001). In order to meet the demand, importing has become the regular practice which accounts to more than sixty per cent of our requirement of petroleum. In the fiscal year 1999-2000 Rs. 58,000 crores was spent on importing petroleum. Diesel and kerosene are also being imported costing Rs. 8.586 crores (Anonymous, 2001), indicating the demand for these natural products. Therefore, it is necessary to search for alternate energy sources which are non petroleum based and renewable such as solar, wind and biomass. Among these, biomass (dedicated energy crops) is the most important and excellent energy source from which fuels of solid, liquid and gaseous nature can be produced. Among the liquid fuels, ethanol is used as an alternative to petroleum (gasohol) by blending with petrol at the rate of 20 per cent (Ramanathan, 2000). Thirty five countries in the world are using 10 per cent alcohol with petrol and 15 per cent alcohol with diesel. The admixture of petrol-alcohol and diesel-alcohol are known as gasohol and diesohol respectively. The alcohol producing countries are USA, Argentina, Brazil, Zimbabwe, India etc. Brazil, at present saves 8 billion dollars per year by blending 20 per cent of ethanol along with petrol. Presently 65 per cent of alcohol is produced by America, 18 per cent by Asia and 15 per cent by Europe (Ahuja, 2001). According to FICCI, our country has the potential to save nearly 80 million litres of petrol annually if 10 per cent alcohol is blended with petrol. Ethanol is an alcohol produced by fermenting sugars present in sugar rich biomass. All over the globe, 60 per cent alcohol is produced from sugar crops, 38 per cent by other crops and only 7 per cent by synthetic means (Ahuja, 2001).

In India, there are 295 distilleries producing 1058 million litres of alcohol as against annual consumption of 1266 million litres (Arbatti, 2001). Nearly 90 per cent of alcohol is produced from molasses, a byproduct of the sugar industry, which is the cheapest feed stock for ethanol fermentation. However, due to the recent hike in the cost of molasses, it has become necessary to search for an alternate biomass substrate for ethanol production. Ethanol production from corn, sweet sorghum and sugar beet have been well established. The other alternate biomass sources tried successfully are the pineapple, cannery waste (Nigam, 1999) and starch (Verma *et al.*, 2000). From the preliminary observations, aqueous extract of deseeded pods of *Samanea saman* showed maximum sugars of 11° brix value indicating its potential for ethanol production (Geeta *et al.*, 2000).

Samanea saman is a tropical tree listed as an economic plant. The trees are cultivated in many parts of India as an ornamental/shade tree particularly on either side of the road. The pods of this tree are harvested in March and April and yields dark brown, straight, thick margined, long, leathery, fleshy pods which are some times used as fodder (Ranjan, 1977) and for the growth of livestock (Jagginavar, 1999). The leaves and the pods of the tree are used as fodder for livestock as green and tender leaves are relished by cattle, sheep, goat and horses. It is a large deciduous tree of 60-80 feet height of which pods are sessile, indehiscent, 6-8 inches long and half to one inch broad, flattened, containing 10-12 seeds embedded in a sugary edible pulp. It is also valued for the shady shelter to delicate plants like vanilla, coffee, cocoa, pepper and nut megs. It thrives best in moist localities and also on dry and barren soils (Wealth of India, 1952). A mature tree can yield about 250-300 kg of pods per season. The ripen pods are available from January to May every year. Pods contain a sugary pulp and form a rich and satisfactory feed for live stock. It was reported that 11.5 litres of absolute ethanol could be produced from 100 kg pods (Wealth of India, 1952). But, the detailed literature on extraction of reducing sugars and other parameters for alcohol production is sparse.

Looking to the potentiality of the substrate, the present investigation was takenup to explicit suitable yeast strains and optimise the parameters for maximum ethanol production with the following objectives.

1. To standardise the pretreatment method for maximum extraction of reducing sugars.
2. To screen the yeasts for utilization of pod extract for ethanol production and to standardise the fermentation conditions for ethanol production.
3. To standardise the pilot-scale production of ethanol from the pods.

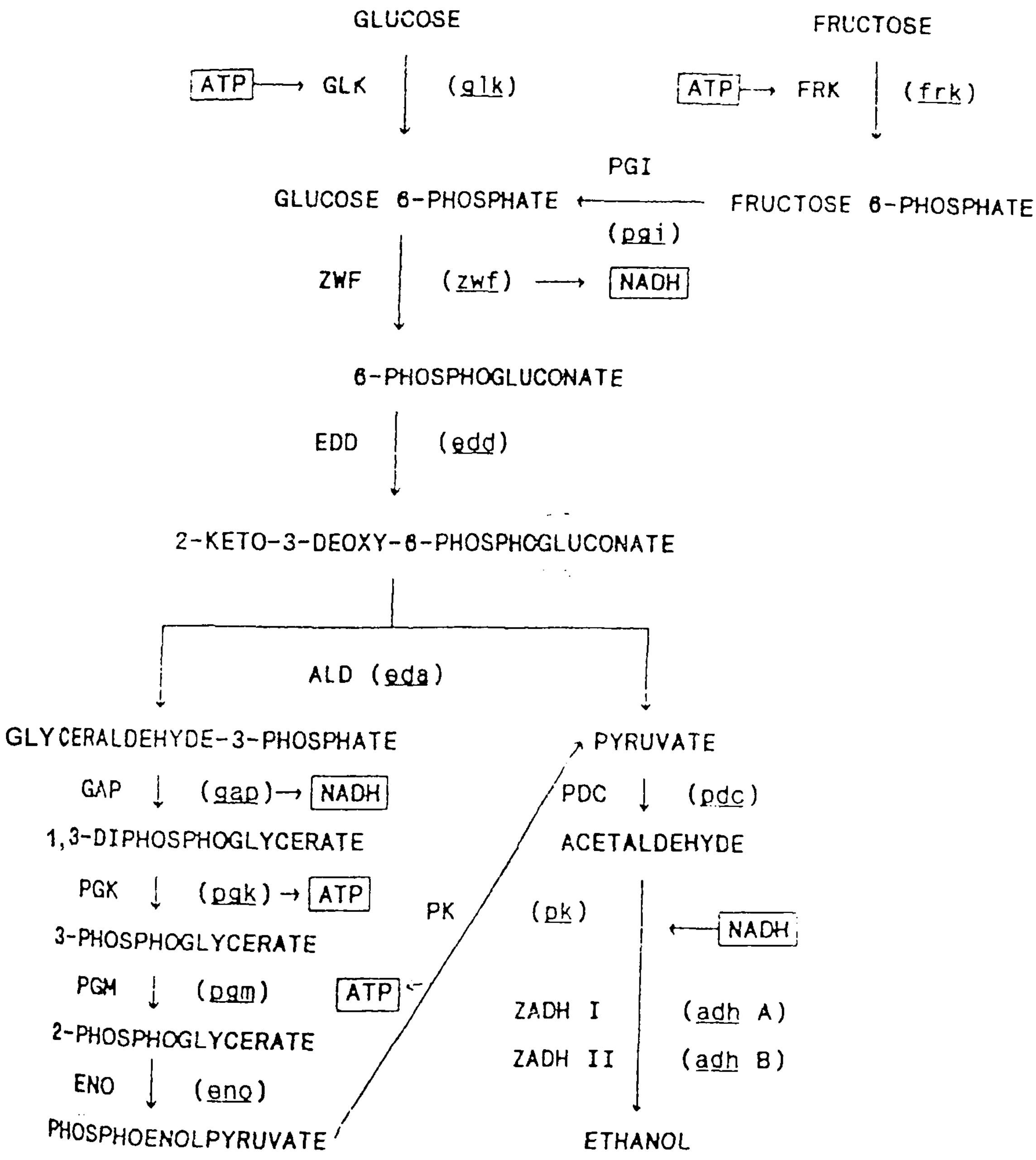
REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The promising renewable resource is the biomass rich in sugars, starch and cellulose and the annual world wide production of cellulose alone accounting to 10^{11} metric tonnes makes it a good substrate for biofuel. Presently, molasses is being used in many of the distilleries for its ideal sugar content suitable for production of ethanol. But, its availability at high cost could increase the cost of production. The major impediment to the development of successful bioconversion process of abundantly available biomass is the physical protection by lignin of cellulose against cellulolytic enzymes. The sugar rich substrates which are easily saccharified such as sugarbeet, sweet sorghum and carob pods have been identified so far as best alternate to molasses. The metabolic pathways adopted by yeasts for utilising various sugars *viz.*, glucose, fructose, sucrose (Gunasekaran *et al.*, 1994) and xylose (Kotter and Ciriacy, 1993) being presented in fig. 1, 2 and 3 respectively. A constant search for alternate sources for ethanol production continues. Several approaches to improve the ethanol production are being employed which include selection of suitable substrate and yeast strains, nutrient supplementation (Geeta *et al.*, 2001) or modifying pH of the medium and identifying optimum temperature (Singh and Jain, 1994), inoculum size and fermentation periods and pilot scale fermentors etc. (Nimbkar *et al.*, 1989). The literature pertaining to these various aspects are being reviewed.

2.1 SUBSTRATES

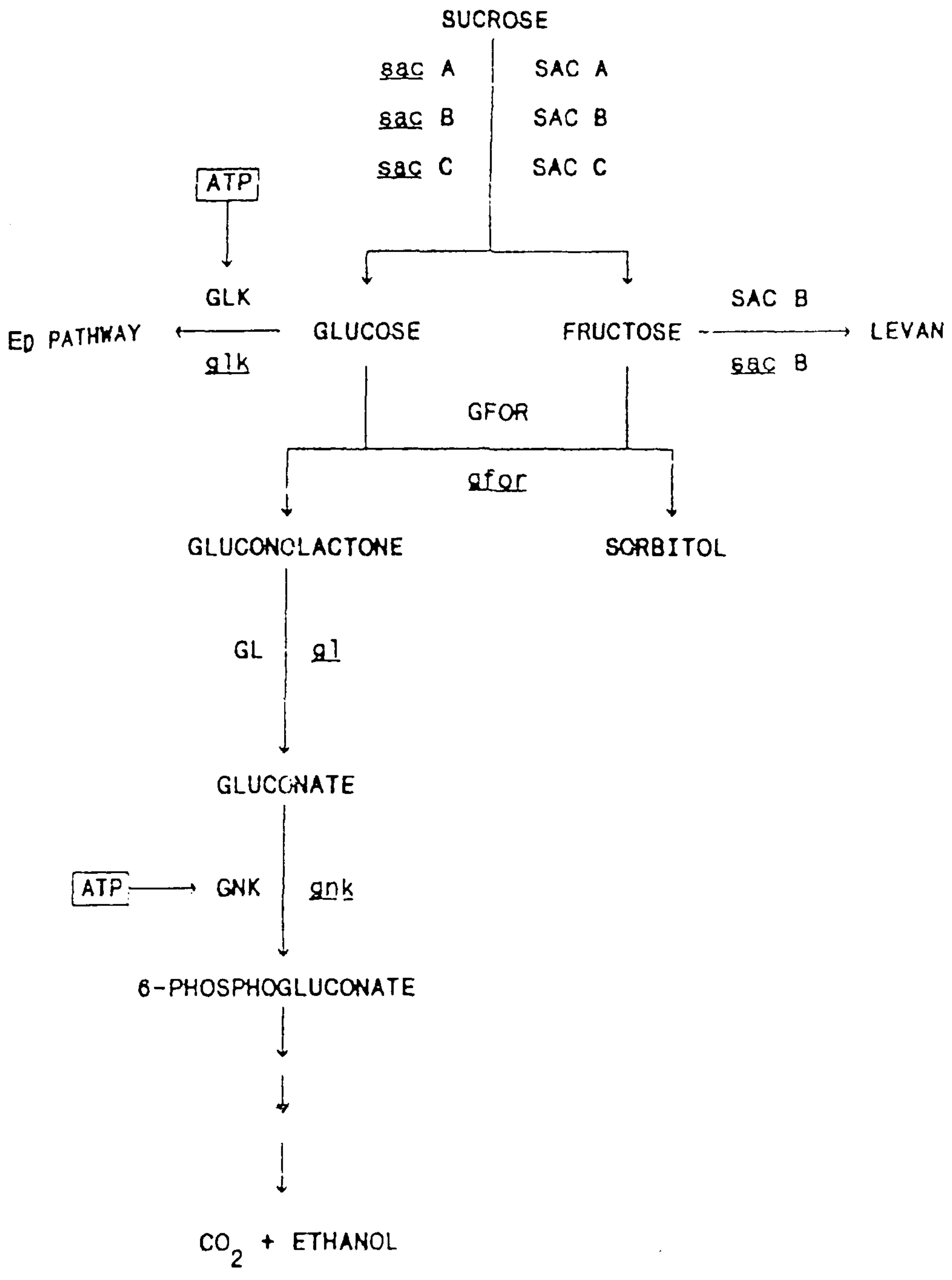
At present molasses is being used for production of ethanol. Molasses contains high amount of reducing sugars. Similar sugars are



glk = Glucokinase
pgi = Phosphoglucose iomerase
edd = 6 phosphogluconate dehydratase
gap = Glyceraldehyde-3-P-dehydrogenase
eno = Enolase
adhA = Alcohol dehydrogenase I
adhB = Alcohol dehydrogenase II

frk = Fructokinase
zwf = Glucose-6-p-dehydrogenase
eda = 2-KDPG aldolase
pgk = Phosphoglycerate mutase
pgm = phosphoglycerate kinase
pdc = pyruvate decarboxylase

Fig. 1. Pathway for glucose and fructose utilisation by yeasts



sac A = Intracellular sucrase
sac C = Extra cellular sucrase
gfor = Glycose – fructose oxido reductase
gl = Glycanolactonase

sac B = Extra cellular levansucrase
glk = Glycokinase
gnk = Gluconate kinase

Fig. 2. Pathway for sucrose utilization by yeasts

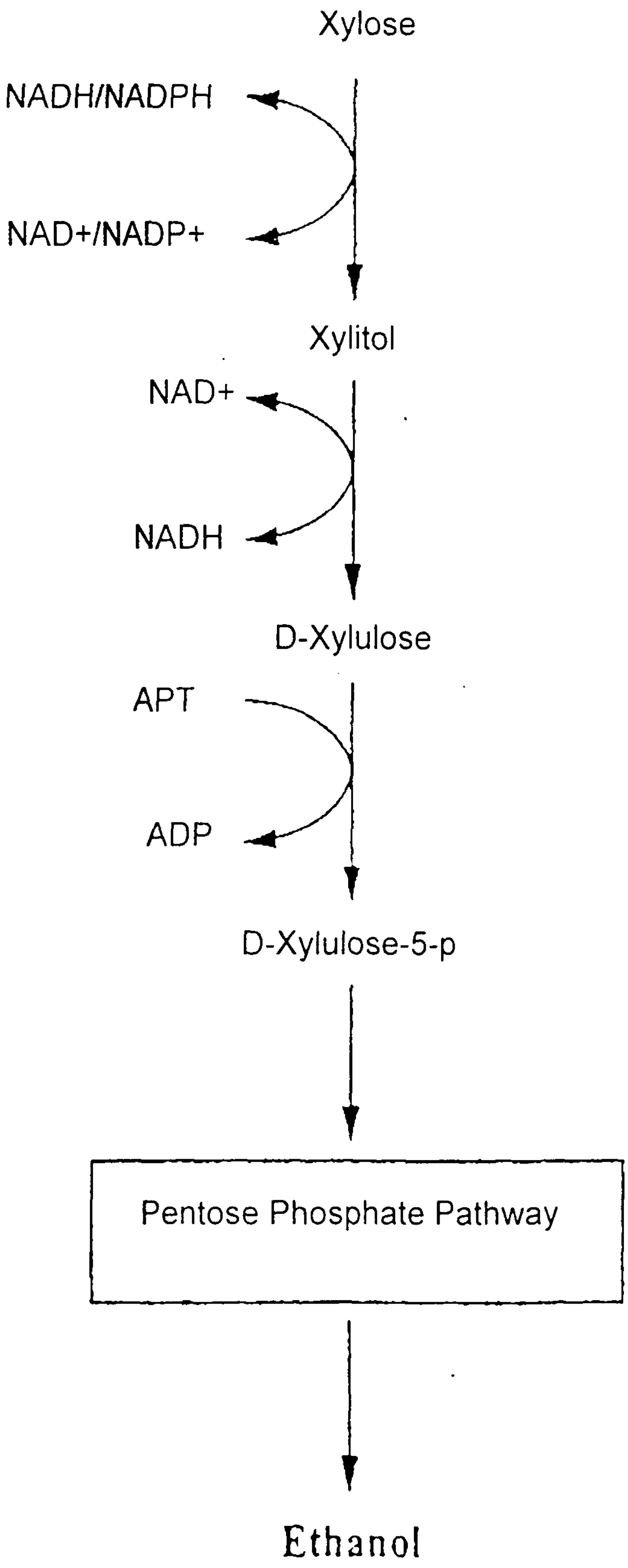


Fig. 3. Pathway for xylose utilisation by yeasts

also being observed in sweet sorghum, beet etc., which can form alternate substrate for ethanol. Likewise many other cellulosic substrates also could be potential substrate for ethanol production as they contain bound sugars. The various biomass substrates having potential are being reviewed here.

Hermann *et al.* (1986) obtained maximum ethanol of 41-42 g L^{-1} from concentrated deproteinized whey having a lactose content of 23 per cent by using *Zymomonas mobilis*, immobilized with sodium alginate. Co-fermentation of sweet sorghum juice and grains was studied by Gibbons and Westby (1989) for production of fuel ethanol and obtained 3.5 per cent (v/v) ethanol from 6.5-7.6 per cent (w/w) reducing sugar after fermentation. Nimbkar *et al.* (1989) successfully fermented unsterilized juice of sweet sorghum by using *S. cerevisiae* strain 3319 and obtained maximum alcohol of 12.45 per cent (v/v).

Pineapple wastes (containing 11.7 per cent soluble sugars) was fermented for ethanol production by Bankoffi and Han (1990) and obtained 0.8 per cent ethanol in 48 hours. Czarnecki and Grajek (1991) studied the influence of temperature and incubation time on starch gelatinization in wheat, rye and maize grain and found that rye starch was the most susceptible to enzymatic hydrolysis and produced highest alcohol yield of 65 per cent. Lynd *et al.* (1991) obtained 400 billion litres of ethanol by microbial conversion of the sugar residues present in waste paper and yard trash and from US land fills. The technical possibilities of the microbial production of ethanol from potato waste using *Clostridium acetobutylicum* DSM 1731 was studied by Grobben *et al.* (1993).

A solid state fermentation process was developed by Pandey (1994) for efficient utilisation of agro industrial residues such as cassava, sugarcane bagasse, sugar beet pulp, coffee pulp or husk and apple pomace for the production of ethanol, single cell protein, enzymes and organic acids. Takahashi *et al.* (1994) assessed the potentiality of sugars *viz.*, glucose (5 g L^{-1}), xylose (80 g L^{-1}) and arabinose (5 g L^{-1}) for biofuel production. They reported that 42.5 g L^{-1} of ethanol was obtained in 96 h, yielding 0.49 g of alcohol per gm of sugar and also reported that the first sugar to be consumed was glucose, followed by arabinose and xylose.

Duff and Murray (1996) attempted bioconversion of forest products industry *viz.*, pulp and paper industry waste cellulosics to fuel ethanol.

Premidevi and Singh (1995) used acid hydrolysate of water hyacinth with 13 per cent total reducing sugar for conversion to ethanol by using *Candida shehatae*. Hammond *et al.* (1996) conducted laboratory studies to assess the ethanol production potential from waste banana *viz.*, whole fruit, pulp, peel and obtained ethanol yield of 0.91, 0.082 and 0.006 L kg^{-1} respectively. Marakis and Marakis (1996) used ripe deseeded carob fruit (*Ceratonia siliqua*) rich in water soluble sugars (54.7%) for production of ethanol and fructose syrup and obtained 4.75 per cent (v/v) and 10.49 per cent (w/w) respectively. Dominguez *et al.* (1997) tested potentiality of corn cobs for biofuel production. Ethanol produced was 5 g L^{-1} hydrolysate.

Suchi Srivastava *et al.* (1997) obtained ethanol from guava pulp by using three different strains of *Saccharomyces cerevisiae*. Shirai *et al.* (1998) cultivated microalgae in the solution from the desalting process of soy sauce waste treatment and utilized the algal biomass for ethanol production of 11 mg g^{-1} (dry cell weight) of *Dunaliella* cells.

Bioethanol from wet oxidised wheat straw was observed by Ahring *et al.* (1999) using *Thermoanaerobacter mathranii*. An attempt to convert spruce and birch to ethanol by using *Saccharomyces cerevisiae* was made by Mohammad *et al.* (1999).

Ethanol yield of 2.90 per cent (v/v) from damaged sorghum and 2.09 per cent (v/v) from rice was obtained by Suresh *et al.* (1999) by simultaneous saccharification and fermentation process. Anantha and Gunasekaran (2000) attempted production of biofuel from liquefied cassava starch and obtained ethanol yield of 21.1 g L^{-1} by batch fermentation process. The conversion of both cellulose (glucose) and hemicellulose (hexose and pentose) for the production of fuel ethanol was intensively studied by Aristidou and Penttila (2000).

Economics of fuel ethanol production from dry milled corn starch using *Zymomonas mobilis* was studied by Krishnan *et al.* (2000) and obtained ethanol yield of 24 g L^{-1} from a 15 million gallon yr^{-1} capacity ethanol plant. The potentiality of pods of *Samanea saman* for ethanol production was revealed by Geeta *et al.* (2001). Mielenz (2001) attempted to commercialize the conversion of sugarcane waste, wheat or rice straw, forestry and paper mill discards, paper portion of municipal waste and dedicated energy crops to ethanol.

Rice straw as a potential feed stock for production of power and fuel was evaluated by Nakamura *et al.* (2001). Ramanathan (2000) obtained an ethanol yield of 42 litres per 100 kg of feed stock by fermentation of root crops namely cassava, potato, yam and sweet potato. Shiva *et al.* (2001) conducted experiment for fermentation of agricultural wastes such as jowar stuffs, jowar stalks and left over corn stalks to ethanol.

2.2 PRETREATMENT METHODS

Most of the vegetative biomass is made up of cellulose, hemicellulose and lignin of which cellulose component is viable source for sugars. Since it is in bound form, the polymer needs external force to break and release the sugars. Therefore, pretreatment methods are employed which may be physical, chemical or biological.

Kahlon and Chaudhary (1988) have compared the rate of hydrolysis of water hyacinth with acid (H_2SO_4 at 15 lb for 15 min) and enzyme (cellulase) and obtained maximum hydrolysis of 25.13 per cent with enzyme and also correlated enzymatic saccharification for maximum ethanol yield than the acid treatment. Pineapple waste was pretreated with cellulases and hemicellulase by Bankoffi and Han (1990) obtained ethanol concentration of 0.8 per cent in 48 hours by fermentation with *S. cerevisiae.*

Dilute acid (H_2SO_4 or HCl at the rate of 0.3 to 2% w/w) was used by Chahal (1991) for hydrolysis of hemicellulose as a pre-treatment method at the temperature of 120 to 180°C from 1 minute to 2 hours or longer and proved that dilute acid is effective in hydrolyzing hemicellulose, beside solubilising a portion of lignin.

Various biomass materials were pretreated with compressed hot water at 200-230°C upto 15 min which solubilized hemicellulose completely beside solubilising 45 and 20 per cent of lignin and cellulose respectively (Mok and Antal, 1992). Sugarcane bagasse pith was pretreated with sodium hydroxide at concentrations 0.1 and 0.2 g g^{-1} pith at 50°C and 80 per cent moisture content and obtained maximum digestibility of 76 and 71 per cent respectively by Vazguez *et al.* (1992). Weil *et al.* (1994) followed steam pretreatment method to hydrolyse the wood chips or wheat straw at high pressure of 250-650 Psi and temperature of 200 to 240°C for 20 minutes.

Corn cob hemicellulose was pretreated with HCl (2%) hydrolysis at 100°C for 2 h after it was subjected to ammonia treatment at 26°C for 24 h. The original lignin content of corn cob was reduced from 0.08 g g^{-1} to 0.01 g g^{-1} after ammonia treatment (Cao *et al.*, 1996). Hammond *et al.* (1996) showed that enzymatic hydrolysis is necessary for maximum yields of ethanol (0.91 L kg^{-1}) from waste bananas (whole fruit, pulp, peel).

Ethanol yield of 4.75 per cent (v/v) was obtained from aqueous extract of ripe deseeded carob fruit with total water soluble sugars of 54.7 per cent consisting of sucrose (77%), fructose (13.9%) and glucose (9%) (Marakis and Marakis, 1996). Dominguez *et al.* (1997) obtained acid hydrolysate comprising mainly xylose from ground corn cobs after dilute hydrochloric acid (2%, w/v) pretreatment at 100°C for 2 hours and achieved specific xylitol productivity of 1.94 g L^{-1} h^{-1} and a xylitol yield of 0.57 g g^{-1} xylose utilized. Weil *et al.* (1998) gave a detailed description of aqueous (liquid hot water) pretreatment method as well as the reactor system used to carry out pretreatment of yellow poplar saw dust.

Ahring *et al.* (1999) investigated the wet oxidation process as a means of solubilising hemicellulose from wheat straw and obtained improved ethanol yield from the acid or commercial enzyme celluclast hydrolysate. An ethanol yield of 0.8 ± 0.008 and 0.05 ± 0.005 g L^{-1} from 0.67 and 2.2 per cent acid (H_2SO_4) treated hydrolysate of wheat straw was obtained and observed highest acid concentration (2.2%) had an inhibitory effect on the ethanol production, whereas the lower acid concentration (0.67%) increased the ethanol production. Berrocal *et al.* (2000) studied the biological upgradation of wheat straw through solid state fermentation with *Streptomyces cyaneus* and achieved 16.4 per cent decrease in lignin content which was attributed to phenol oxidase enzyme which was produced during the growth of bacteria.

Pretreated peanut shells with 1 per cent H_2SO_4 at 103.5 k.pa for 2 h which resulted in extraction of xylose (50 g L^{-1}), glucose (3.5 g L^{-1}), galactose (5 g L^{-1}), mannose (1 g L^{-1}) and arabinose (7 g L^{-1}) (Chandrakant and Bisaria, 2000). Lee *et al.* (2000) could achieve 82 per cent hydrolysis of cellulose and near total deploymerization of xylose to yield a solution with 4 per cent sugar by pretreatment of cellulosic biomass with 0.07 per cent sulphuric acid.

An effective method was developed by Nikolov *et al.* (2000) for production of glucose, using enzymatic hydrolysis of waste cellulose fibers by the cellulase complex from *Trichoderma reesei* after pretreatment with H_3PO_4 (0.25%), which resulted in 80 per cent degradation of the substrate. *Aspergillus* and *Bacillus* species were used as source of enzymes at 5 per cent for hydrolysis of yam by Ramanathan (2000) and was able to obtain maximum reducing sugar content of 84 g L^{-1} with 74.3 per cent efficiency. Bishnoi *et al.* (2001) pretreated paddy straw (3.5-5.0% lignin) with 28 per cent EDA (Ethylene diamine) and achieved 72.41 per cent decrease in lignin content.

Geeta *et al.* (2001) optimized the extraction of soluble reducing sugars from the *Samanea saman* pods by hot water and acid extraction and observed maximum release of reducing sugars (313 mg g^{-1}) at one per cent acid (H_2SO_4) concentration.

Diluted acid (70-77%) at the ratio 1.25:1 was introduced as pretreatment agent to remove the hemicellulose content of biomass before decrystallisation and hydrolysis of the cellulose fraction (Jacobus and Wyk, 2001). Nakamura *et al.* (2001) carried out the enzymatic saccharification of exploded rice straw using mixed enzymes (cellulases) such as meicelase and acucelase for the efficient conversion of rice straw into sugars and obtained ethanol yield of 86 per cent (w/w) by fermentation with *Pichia stipitis.* Shiva *et al.* (2001) compared acid hydrolysis (HCl) and enzyme (amylases and amylo-glucosidases) pretreatment methods for starchy substrates and recorded maximum extraction of sugars (0.38 g g^{-1} of sample) by enzyme hydrolysis as compared to acid hydrolysis.

Geeta *et al.* (2002b) studied the relative efficiency of different fungal cultures (*Aspergillus foetidus, A. niger, Phanerochaete chrysosporium* and *Trichoderma viride*) on the degradation and saccharification of paddy straw for bioethanol production. Among fungal cultures, *P. chrysosporium* was found to be superior in releasing fermentable sugars which yielded 534 mg ml^{-1} and accounts to 53.40 per cent, followed by *T. viride* and *A. niger* which yielded 499 mg ml^{-1} and 401 mg ml^{-1} equivalent to 49.90 and 40.10 per cent respectively. Similarly, per cent loss in total solids was also maximum in substrates inoculated with *P. chrysosporium, T. viride* and *A. niger* which accounts to 24.0, 19.5 and 17.5 per cent loss in total solids as compared to uninoculated control and that inoculated with *A. foetidus.*

Sugarcane bagasse was pretreated by Mark *et al.* (2002) along with hot water at temperature range of 170 to 230°C for 1 to 46 minutes and achieved ≥80% xylan recovery with ≥80% conversion by simultaneous saccharification and fermentation. Reducing sugars of 16-28 per cent was obtained by Singh *et al.* (2002) after sequential hydrolysis of slurried substrates (30-40%) *viz.*, tapioca powder, corn and barley with biotemptase and amylo 300.

2.3 STRAINS OF YEAST FOR ETHANOL PRODUCTION

Nigam *et al.* (1985) studied 11 strains of undescribed species of *Clavispora* for fermentation of D-xylose under aerobic conditions and reported maximum ethanol concentration and volumetric ethanol productivity of 10.9 g L^{-1} (57.2% of theoretical yield) and 0.11 g L^{-1} respectively with initial D-xylose concentration of 40 g L^{-1}.

Nimbkar *et al.* (1989) screened sixteen yeast strains for their ability to ferment sweet sorghum juice and obtained maximum ethanol of 12.45 per cent (v/v) from *S. cerevisiae* strain 3319. Bertolin *et al.* (1991) selected *Saccharomyces cerevisiae* var. *uvarum* among 8 new osmotolerant yeast strains collected from Brazilian alcohol factories for alcoholic fermentation. Gunasekaran and Kaimini (1991) achieved higher ethanol productivity from lactose by immobilized cells of *Kluyveromyces fragilis* and *Zymomonas mobilis*. Ferrari *et al.* (1992) evaluated *Pichia stipitis* NRRLY 7124 for production of ethanol.

The thermotolerant alcohol producing yeast strain *Kluyveromyces marxianus* IMB3 was studied by Fleming *et al.* (1993). The culture grew on media containing 10 per cent sucrose (w/v) at 45°C and was able to produce ethanol concentrations of 33 g L^{-1}. Kotter and Ciriacy (1993) studied xylose utilization of *Saccharomyces cerevisiae* transformant that expressed two key enzymes (xylose reductase and xylitol dehydrogenase) derived from *P. stipitis* in xylose metabolism.

Maximum alcohol concentration of 5.43 to 6.35 (v/v) was obtained by Saigal (1994) by strains of *K. marxianus* and *Hansenula* at 43°C in 48 hours of fermentation period. Sohn and Seu (1994) achieved maximum ethanol concentration of 86.6 g L^{-1} (85% theoretical) by strains of *Kluyveromyces marxianus* from 20 per cent (w/v) glucose medium at 40°C in 96 hours of fermentation period. Abate *et al.* (1996) tested pure and mixed cultures of *Zymomonas mobilis* and *Saccharomyces cerevisiae* sp. for the production of ethanol by fermentation medium containing sucrose (200 g L^{-1}) at 30°C and obtained ethanol yield of 0.5 g g^{-1} (1.5 g L^{-1}) of sugar consumed in 63 hour fermentation by mixed culture.

Co-immobilized cultures of *S. cerevisiae* and *C. shehatae* which could convert glucose and xylose simultaneously to ethanol yield of 0.48 g g^{-1} total sugars was studied by Lebeau *et al.* (1997). Scory and Bothast (1997) screened nineteen *Aspergillus* and ten *Rhizopus* strains for their ability to ferment simple sugars (glucose, xylose and arabinose) as well as complex substrates (cellulose, oat-spel+ xylan, corn fiber and corn germ pressing), of which three *Rhizopus* strains were produced more than 31 g L^{-1} of ethanol by 72 h and glucose, xylose, cellobiose and corn fiber were fermented with perspective yields of 100, 47, 80 and 40 per cent of theoretical yield. Suchi Srivastava *et al.* (1997) used three isolates (isolate 1, 2 and MTCC 1972) of *S. cerevisiae* for ethanol production from guava pulp and obtained maximum ethanol yield of 5.8 per cent (w/v) by isolate-2 compared to other two isolates.

The fermentation of hydrolysate using *Thermoanaerobacter mathranii* strain A3M1 which can grow on undiluted wood hydrolysate and simultaneously produce ethanol was observed by Ahring *et al.* (1999). Burdette *et al.* (1999) developed a mutant strain (39E H8) of *Thermoanaerobacter ethanolicus* that displayed 8 per cent (v/v) ethanol tolerance for growth compared to wild type (39E).

The fermentation characteristics of *Saccharomyces cerevisiae* strains which over expresses a constitutive OLE1 gene which is necessary for enhancing the ethanol productivity was studied by Kajiwara *et al.* (1999). Suresh *et al.* (1999) utilized *Aspergillus niger* (NCIM 1248) and *Saccharomyces cerevisiae* VSJI, for simultaneous saccharification and fermentation of grains and obtained ethanol yield of 2.90 per cent (v/v).

A mixed culture was developed by Anantha and Gunasekaran (2000) consisting of an amylolytic yeast strain *S. diastaticus* and *Z. mobilis* which improved ethanol production (0.34 g g^{-1}) as compared to monoculture (0.24 g g^{-1}) of yeast. Lopez Contreras *et al.* (2000) studied *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol (ABE) from saccharides of domestic organic waste (4 g ABE/100 g of DOW). More than 40 yeast strains were screened by Sreenath and Jeffries (2000) of *P. stipitis* and *C. shehatae* to determine their fermentation rates on mixed sugars. All of the tested strains fermented both glucose and xylose and attained ethanol concentration of 34.8±2.42 g L^{-1} with *P. stipitis* and 34.0±1.67 g L^{-1} with *C. shehatae.*

Ethanol production by coculture was studied by Verma *et al.* (2000) using *S. diastaticus* and *S. cerevisiae* 21 in raw unhydrolysed starch which yielded ethanol of 48 per cent higher (24.8 g L^{-1}) than that obtained with the monoculture of *S. diastaticus* (16.8 g L^{-1}). Bajaj *et al.* (2001) screened twenty two strains of yeast, isolated from natural sources for ethanol production, of these 5 isolates were found to produce ethanol in significant amounts. These strains were examined for desired fermentation characteristics like ethanol production from molasses, ethanol tolerance, growth rate, respiratory deficiency level and phenotype with respect to killer, sensitive or neutral characters. Two isolates SBS13 and SBS14 were found to be of desired properties for exploiting at commercial level.

Geeta *et al.* (2001) screened, standard and local yeast strains for ethanol production from pods of *Samanea saman* and obtained maximum ethanol concentration of 40.43 g L^{-1} by local isolate BCY-107 followed by BCY-108 (36.06 g L^{-1}). Four strains of *Zymomonas mobilis* were screened by Panesar *et al.* (2001) for their ability to produce ethanol from molasses medium at pH 6.0. Ramakrishna *et al.* (2001) screened five fructose negative mutants (fru) (KLRI, KLR2, KLR3, KLR4 and KLR5) of *Zymomonas mobilis* NCIM 2915 for production of fructose syrup and ethanol from water soluble carob sugars and obtained maximum ethanol and fructose yield by KLR4 mutant (120 g L^{-1} and 4% w/v) at 120 h of fermentation.

Two *Erwinia* endoglucanase genes were cloned (Zhou *et al.*, 2001) into an ethanol producing *Klebsiella* species, resulted in an organism with a capacity to produce 22 per cent more ethanol when fermenting crystalline cellulose synergistically with added fungal cellulases.

2.4 RESIDUAL SUGAR CONTENT

Nimbkar *et al.* (1989) studied the sugar utilization by the yeasts during fermentation of unsterilized juice of sweet sorghum with 20 per cent fermentable sugars at 48, 72 and 120 hours and observed decrease in the sugar content to 2-4 and 1 per cent at 48 and 72 to 120 hours respectively.

The residual sugar concentration of 13.47 g L^{-1} was observed by Singh and Jain (1994) after fermentation of 100 g L^{-1} sucrose medium yielding 38.54 g L^{-1} of ethanol by *Z. mobilis* strain NRRLB-4286. Ryn *et al.* (1994) obtained 64.3 g L^{-1} of ethanol by utilising 94 per cent of 150 g L^{-1} soluble starch with a mixed culture and the starch content in the damaged grains used was lower by 30 per cent and 40 per cent when compared with the fresh grains of sorghum.

A maximum alcohol concentration of 4.75 per cent (v/v) with 105.1 g L^{-1} of residual sugar was obtained by Marakis and Marakis (1996) during 100 h of fermentation of aqueous carob extract which contained 203 g L^{-1} total sugar. Verma *et al.* (2000) obtained ethanol yield of 21.8 g L^{-1} from initial starch concentration of 100 g L^{-1} by coculture of *S. diastaticus* and *S. cerevisiae* 21 and obtained residual starch content of 22 per cent.

2.5 FERMENTATION TIME

Zossi *et al.* (1990) performed ethanol fermentation of sugarcane juice for 7 days at pH 6.0 with supplementation of SO_2 (700 ppm) and achieved fermentation efficiency of 96.916±0.83 per cent. Marakis and Marakis (1996) studied the effect of six different fermentation periods *viz.*, 0, 24, 48, 72, 90 and 100 hours on ethanol production from aqueous carob extract and achieved maximum alcohol concentration of 4.75 per cent (v/v) at 100 hours of fermentation period.

Ethanol fermentation from *Dunaliella* microalgae at 25°C was within (Shirai *et al.*, 1998) 5 days and were able to obtain ethanol yield of 11 mg g^{-1} of substrate. An experiment was conducted for conversion of corn starch to fuel ethanol which was 72.2 g L^{-1} ethanol produced in 120 minutes residence time (Krishnan *et al.*, 1999). Damaged sorghum and rice grains were utilized by Suresh *et al.* (1999) for ethanol production and obtained ethanol yield of 2.90 per cent v/v at 30°C, after 5 days of fermentation. A maximum alcohol yield in 3 days during fermentation of yam to ethanol by *Saccharomyces cerevisiae* was observed by Ramanathan (2000).

Sreenath and Jeffries (2000) fermented wood hydrolyzate using yeasts for 3-5 days at temperature and pH of 25-27°C and 5.5 to 7.0 respectively, and obtained average ethanol concentration of 34.8 ± 2.42 g L^{-1}. The effect of four different fermentation periods *viz.*, 24, 48, 72 and 96 hours on ethanol production from starch medium was studied by Verma *et al.* (2000). A maximum ethanol concentration of 24.8 g L^{-1} at 48 hours was achieved as compared to 13.7 and 21.6 g L^{-1} at 24 and 96 hours respectively. Ramakrishna *et al.* (2001) obtained maximum ethanol yield of 120 g L^{-1} at 120 h of fermentation from water soluble carob sugars by mutant strain of *Zymomonas mobilis* KLR 4.

2.6 EFFECT OF pH ON ETHANOL YIELD

Nimbkar *et al.* (1989) obtained maximum alcohol concentration of 12.45 per cent (v/v) at pH 4.5 from fermentation of unsterilized juice of sweet sorghum by *S. cerevisiae* strain 3319.

A maximum ethanol concentration of 79.1 ml L^{-1} from cane molasses at operating variables of pH 4.2, temperature 32°C in 30 days of operation was obtained by Garcia and Suarez (1992). Marakis and Marakis (1996) obtained maximum alcohol concentration of 5.8 per cent (v/v) at pH 4.5 from aqueous carob extract after 120 hours of fermentation.

Suchi Srivastava *et al.* (1997) showed that the optimum, initial pH of guava pulp medium for the production of ethanol was 5.0 for all three yeast strains employed and obtained maximum ethanol yield of 5.8 per cent (w/v) during 36 hours fermentation of guava pulp by isolate-2 and standard culture of *S. cerevisiae*. The rate of ethanol production from wood hydrolyzates was enhanced by pH in the range of 5.1-7.0. Ethanol production was maximum (20 g L^{-1}) at pH 6 and hydrolysate having pH 7.7, produced 30 per cent less ethanol (Sreenath and Jeffries, 2000).

2.7 EFFECT OF TEMPERATURE AND ETHANOL YIELD

Hermann *et al.* (1986) proved that, optimal growth temperature and pH for maximum ethanol production (41-42 g L^{-1}) are 30°C and 5.0 respectively during whey fermentation by *Zymomonas mobilis*. Nimbkar *et al.* (1989) studied the effect of three different incubation temperatures *viz.*, 25, 30 and 35°C on the ethanol production from unsterilized juice of sweet sorghum with *S. cerevisiae* and obtained maximum alcohol of 12.45 per cent (v/v) at 30°C and also observed inconsistent alcohol production at 35°C due to a temperature shock.

A maximum ethanol concentration of 88 g L^{-1} from 200 g L^{-1} sucrose medium at optimum temperature of 30°C and pH 6.0 with ethanol yield of 0.48 g g^{-1} was obtained by Singh and Jain (1994). An ethanol concentration of 91 g L^{-1} and productivity 2.7 g L^{-1} h^{-1} at the higher temperature of 35°C was achieved by Lother and Oetterer (1995) in a molasses medium containing 22 per cent (w/v) total sugar when thermotolerant strain (KF7) of *S. cerevisiae* was used. Abate *et al.* (1996) obtained ethanol yield and an average hourly productivity of 0.5 g g^{-1} sugar consumed and 1.5 g L^{-1} respectively in 14 hours after inoculation of *S. cerevisiae* at temperature of 30°C from 200 g L^{-1} of sucrose.

Both simultaneous saccharification and fermentation (SSF), and separate hydrolysis and fermentation (SHF) in the fluidized bed reactor (FBR)system was studied by Krishnan *et al.* (1999). The hydrolysis and fermentation steps were performed at their optimum temperatures of 55 and 30°C, respectively and were able to obtain volumetric ethanol productivity of 19-25 g L^{-1}. Chandrakant and Bisaria (2000) carried out simultaneous isomerisation and fermentation (SIF) of xylose and simultaneous isomerisation and cofermentation (SICF) of a glucose/xylose mixture by *Saccharomyces cerevisiae* in the presence of xylose isomerase by addition of borate to the medium which resulted in ethanol concentration and metabolic yield of 29.8 g L^{-1} and 0.42 respectively and temperature modulation from 30 to 35°C during fermentation further enhanced the above parameters to 39 g L^{-1} and 0.45 respectively.

The effect of five different temperatures *viz.*, 25, 30, 35, 37 and 40°C on the ethanol production from starch (100 g L^{-1}) was studied by Verma *et al.* (2000). They observed that maximum ethanol concentration of 21.8 g L^{-1} was at optimum temperature of 30°C in 48 hours of fermentation period. Panesar *et al.* (2001) optimized the fermentation parameters *viz.*, temperature, pH and inoculum level as 30°C, 6.0 and 10 per cent (v/v) respectively for ethanol production from molasses medium.

2.8 EFFECT OF INOCULUM SIZE ON ETHANOL PRODUCTION

Gibbons and Westby (1986) studied the effects of inoculum size on solid phase fermentation of fodder beets for fuel ethanol production and proved that 5 per cent of inoculum of *S. cerevisiae* (w/w) resulted in rapid ethanol (9% v/v) production. Increase in the inocula indicated no advantages, and lower inocula resulted in increased fermentation time.

An alcohol content of 2.18 per cent (w/v) from whey inoculated with 1.00 per cent of *S. cerevisiae* at pH 4.0 and temperature of 22°C after 18 hours of fermentation period was obtained by Mathur *et al.* (1986). The highest alcohol recovery of 52 per cent by adding yeast inoculum at the rate of 10 per cent (v/v), during fermentation of sweet sorghum juice was achieved by Swaminathan *et al.* (1987).

The effect of inoculum size on fermentation of whey for fuel ethanol production was studied by Cuesta and Cornejo (1988) and proved that increasing the inoculum level from 1.8 million to 1.9 million cells per ml decreased the time taken to maximum fermentation (about 73% theoretical value) from 360 to 84 h. The cultural conditions was optimised by Kahlon and Chaudhary (1988) for water hyacinth for maximum alcohol production which required 8 per cent yeast inoculum and 36 h fermentation period. Nimbkar *et al.* (1989) studied the effect of different inoculum levels *viz.*, 2, 4, 6, 8 and 10 per cent on the ethanol production from unsterilized juice of sweet sorghum and obtained maximum alcohol concentration of 12.45 and 12.23 per cent (v/v) at 6 and 2 per cent respectively, and also reported that, 2 per cent inoculum level appears to be sufficient for alcohol production.

Mendoza and Raymundo (1990) optimized the fermentation conditions for batch alcohol production from molasses. The parameters selected were, inoculum size, temperature, initial sugars and pH which resulted as 10 per cent (v/v), 30°C, 10 per cent and 5.5 respectively and observed that fermentation efficiency and ethanol yield of 83.3 to 92.6 and 4.6 per cent respectively. A maximum ethanol production of 88 g L^{-1} from 200 g L^{-1} sucrose medium at 10 per cent inoculum size in 16 to 18 hours was obtained by Singh and Jain (1994). Ramanathan (2000) could achieve maximum alcohol recovery of 5.85 per cent at (5%) yeast concentration during fermentation of yam to ethanol.

2.9 EFFECT OF NUTRIENT SUPPLEMENTATION ON ETHANOL PRODUCTION

Swaminathan *et al.*(1987) achieved highest alcohol recovery of 52 per cent by supplementation of sweet sorghum juice (4.5 pH) with 2 per cent ammonium sulphate after 48-62 hours of fermentation. Cuesta and Cornejo (1988) proved that, addition of equal amounts of H_3PO_4 and $(NH_4)_2SO_4$ upto 0.7 per cent each increased the fermentation rate and level of ethanol production and addition of 0.3 per cent of each of these salts to 0.5 per cent yeast extract had an even greater effect during the whey fermentation by *Candida pseudotropicalis* IFI 268. Nimbkar *et al.* (1989) supplemented unsterilized sweet sorghum juice with 2 per cent ammonium sulphate during fermentation by *S. cerevisiae* and able to obtain only 9.05 per cent (v/v) of ethanol concentration as compared to unsupplemented juice (9.08% v/v).

Patil *et al.* (1989) obtained 20-30 per cent more ethanol in the presence of novel supplements *viz.*, skim milk, chitin and waste mycelium individually or in combination at 30°C with 15 per cent fermentable sugars by *S. cerevisiae* and also the efficiency of the process was improved from 66 to 87 per cent. Cane molasses (18% total sugars) at pH 4.7 was fermented for 40 hour by *S. cerevisiae* with ammonium sulphate (1.5 g L^{-1}) and other N sources *viz.*, yeast extract and Difco-Bi-Tek. Efficient sugar utilization was with ammonium sulphate (Huertas, 1990).

The influence of sulphur dioxide (700 ppm) on fermentation of sugarcane juice by distillery yeast at pH 6.0 was studied by Zossi *et al.* (1990). They obtained fermentation efficiency of 96.916±0.83 per cent. Dubey *et al.* (1991) studied the effect of hormonal growth factor formulations containing oestrogen, progesteron, oxytocin and lecithin complex on the fermentation efficiency of distillery yeast during fermentation of molasses medium at 24, 48, 60 and 72 h. It was reported that all the growth factors had a stimulatory effect on fermentation with fermentation efficiency of 94.72, 93.51, 94.92 and 89.67 per cent at 2 ppm concentration in 60 hour fermentation period compared to that of control (79.15% in 72 h).

The effect of supplementation of ammonium sulphate on ethanol fermentation from molasses medium at concentrations of 0.1 per cent and 1 g L^{-1} using *Z. mobilis* strain B-61147 was studied by Iida *et al.* (1993). They obtained ethanol concentration and hourly productivity of 65 to 70 g L^{-1}, 58.4 g L^{-1} and 75.1 g L^{-1}, 68.8 g L^{-1} respectively, at 0.1 per cent and 1 g L^{-1} $(NH_4)_2SO_4$, compared with 43.9 g L^{-1} for *S. cerevisiae* at 0.5 per cent concentration at pH and temperature 4.8 and 30°C respectively. Amartey and Jeffries (1994) showed that addition of inexpensive supplement such as corn steep liquor (CSL) as a substitute for expensive nitrogen in pentose fermentation mediated by strains *P. stipitis* and *C. shehate* had increased the ethanol concentration to 26.5 g L^{-1} in 205 hours of fermentation. Marakis and Marakis (1996) supplemented aqueous carob extract with ammonium sulphate and sodium dihydrogen phosphate at the rate of 0.3 and 0.1 per cent respectively during fermentation by *S. cerevisiae* and obtained maximum ethanol concentration of 5.8 per cent (v/v) after 120 hours of fermentation.

A maximum ethanol yield of 30.8 g L^{-1} was obtained when the grape skin pulp extract was supplemented with NH_4NO_3 (0.1%) and K_2HPO_4 (0.1%) compared to ethanol yield (23.3 g L^{-1}) without supplementation (Israilides *et al.*, 1998). Anantha and Gunasekaran (2000) obtained higher ethanol yiled of 44.2 and 54.9 g L^{-1}, respectively by supplementation of liquefied cassava starch with ammonium sulphate (1 g L^{-1}) and yeast extract (10 g L^{-1}) which was higher than that of without supplementation (36.5 g L^{-1}) in a mixed culture of *Z. mobilis* and *S. diastaticus.*

Each tonne of cassava requires 200 g of triple superphosphate and ammonium sulphate each and 20 g of Magnesium sulphate also for fermentation of cassava to ethanol (25-30%) (Ramanathan, 2000). An addition of zinc sulfate ($ZnSO_4$) @ 10 mg L^{-1} in the fermentation medium of wood hydrolysate increased the rate of sugar utilization and ethanol yield due to high activity of zinc dependent alcohol dehydrogenase and, obtained higher ethanol yield of 26-30 g L^{-1} (0.33 to 0.49 g g^{-1}) (Sreenath and Jeffries, 2000). Geeta *et al.* (2001) studied the effect of zinc sulphate ($ZnSO_4$) at various concentrations *viz.*, 0, 10 and 15 mg L^{-1} on ethanol production from the pods of *Samanea saman* and obtained *maximum ethanol concentration at 10 mg* L^{-1} $ZnSO_4$.

Shiva *et al.* (2001) conducted studies by supplementing cholesterol, soya lecithin, oleic acid and tween 80, individually and their combinations, in the fermentation medium used for ethanol production and found that, of all the supplements tested, combination of cholesterol and oleic acid could produce 19.74 per cent enhanced yields as compared to unsupplemented control. Ramakrishna *et al.* (2002) carried out investigations to determine alcohol and fructose syrup at different temperature levels employing *Z. mobilis* (KLR5) and *Arthrobacter* sps. on carob pod syrup. Maximum alcohol and fructose syrup of 4.2 per cent and 126 g kg^{-1} respectively was observed in *Z. mobilis* followed by *Arthrobacter* (4.1% and 122 g kg^{-1}) at 35°C.

Sridhar and Rao (2002) studied the influence of nitrogen sources (ammonium sulphate, ammonium chloride, urea, casein hydrolysate, yeast extract and peptone) and organic ions (magnesium, calcium, manganese, zinc and potassium) at different concentrations on thermotolerance, osmotolerance and ethanotolerance of *Saccharomyces cerevisiae* VS3 at 30 and 42°C and obtained enhanced ethanol yield of 12 and 8 g L^{-1} at 30°C when medium was supplemented with yeast extract and ammonium sulphate respectively.

2.10 TYPE OF FERMENTORS FOR ETHANOL PRODUCTION

Hermann *et al.* (1986) fed the deproteinized whey with lactose content of 23 per cent continuously to 3 litre fermentor bottle at a flow rate of 25 ml h^{-1} with *Zymomonas mobilis* immobilized with sodium alginate and obtained ethanol yield of 41-42 g L^{-1}. Saucedo and Karim (1996) carried out fed-batch mode of fermentation with 17 per cent (w/v) xylose medium at temperature and pH of 34°C and 6.4 respectively, and obtained ethanol productivity of 1.8 g L^{-1} after 40 hours of fermentation. Sun *et al.* (1997) demonstrated high productivity of ethanol from soluble starch by simultaneous saccharification and fermentation (SSF) in a laboratory scale fluidized bed reactor (FBR) using a co-immobilized biocatalysts of glucoamylase from *S. cerevisiae* and *Z. mobilis*.

Batch and fed-batch fermentations were examined in a lab scale (3.3 L) anaerobic bioreactor. Fed-batch technique with a suitably adjusted feed rate was possible to completely ferment the glucose and mannose sugars (Mohammad *et al.*, 1999). Krishnan *et al.* (2000) studied the economics of fuel ethanol production from dry milled corn starch in fluidized bed reactors (FBRs) using immobilized biocatalysts.

Ethanol production on a pilot scale for the conversion of high solids saccharified corn mash to ethanol by continuous fermentation and carbon dioxide stripping was demonstrated by Taylor *et al.* (2000). Verma *et al.* (2000) carried out a single step fermentation process for ethanol production by using monoculture and coculture of amylolytic yeasts and *S. cerevisiae* and able to obtain higher ethanol yield in 48 hours of fermentation at 30°C using 60 g L^{-1} starch in batch fermentation by cocultures.

An alcohol fermentation of an enzymatic hydrolysate of steam exploded rice straw in a membrane bioreactor coupled with a pervaporation system was studied by Nakamura *et al.* (2001). They reported an ethanol yield of 86 per cent (w/w), reaching 50 g dm^{-3} in a bioreactor.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The trees of *S. saman* planted as road side ornamental plants are widely spread in the campus of college of Agriculture, University of Agricultural Sciences, Dharwad. The mature pods of *S. saman* which fall in the month of March and April were collected and used as a substrate for the study.

The details of material used and methods employed during course of investigation are presented in this chapter.

3.1 BIOCHEMICAL CONSTITUENTS OF SUBSTRATE

The matured pods of *S. saman* were mechanically deseeded and broken into approximately one centimeter bits and were used for extraction of juice. The biochemical constituents such as total sugar, reducing sugar, total solids, organic carbon, phenols, tannin, pH, nitrogen and protein of raw deseeded pods were analysed by following standard procedures (Sadasivam and Manickam, 1992).

3.2 PRETREATMENT OF THE SUBSTRATE FOR EXTRACTION OF REDUCING SUGARS

The different pretreatment methods employed for extraction of maximum reducing sugars are as stated below.

3.2.1 Physical pretreatment methods (Geeta *et al.*, 2000)

3.2.1.1 Aqueous extraction without heat treatment

The bits of (1 cm) deseeded pods were soaked in distilled water in the ratio 1:6 (substrate : distilled water) (w/v) at room temperature for 10 h with intermittant shaking and were analysed at hourly intervals for reducing sugars.

3.2.1.2 Aqueous extraction with heat treatment

The deseeded pods of 1 cm bits were soaked in water in the ratio of 1:6 (substrate:distilled water) and the samples were incubated at 50, 80 and 100°C for 10 h. The samples were analysed at hourly intervals for reducing sugars.

3.2.2 Biological pretreatment

Five grams of deseeded bits of *S. saman* pods were transferred to 250 ml conical flask and inoculated with different fungal cultures. The fungi *Aspergillus foetidus* (NCIM 515), *A. niger* (NCIM 616), *A. awamori* (IARI) and *A.niger* (local isolate) obtained from NCIM Pune and IARI New Delhi respectively, except *A. niger* which is a local isolate and maintained on potato dextrose agar (PDA) medium (Tuite, 1969) (Appendix-I) were used for pretreatment. The cultures were grown on agar medium in plates. Agar disks @ 4 were inoculated to each flask both under sterile and unsterile substrate and incubated for five days at ambient temperature. The sugars were extracted by adding distilled water at the ratio of 1:6 (substrate: distilled water).

The effect of biological pretreatment was evaluated in terms of per cent loss in weight which was estimated by taking the difference in initial weight and final weight of the samples as given below.

$$\text{Per cent loss in weight} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

3.2.3 Chemical pretreatment methods

3.2.3.1 Acid pretreatment

The deseeded bits of pods of *S. saman* were soaked in sulphuric acid at concentrations of 0.10, 0.50, 1.00 and 1.50 per cent along with a control (where only distilled water was added) in the ratio 1:6 (Substrate:diluted sulphuric acid) and incubated for 10 hours under agitated conditions. The reducing sugars released were estimated at hourly intervals.

3.2.3.2 Alkali pretreatment

The deseeded bits of *S. saman* pods were soaked in sodium hydroxide at concentrations of 0.50, 1.00 and 1.50 per cent along with a control (with only distilled water) at the ratio of 1:6 (substrate:Alkali concentration w/v) and incubated for 10 hours with agitation at ambient temperature and the reducing sugars were estimated at hourly intervals.

3.3 SCREENING AND SELECTION OF EFFICIENT YEAST CULTURES FOR ETHANOL PRODUCTION

The best pretreatment method obtained was 1.5 per cent acid pre-treatment which released maximum reducing sugars (35.26 g L^{-1}). This was finally used in all the following experiments.

The twenty yeast cultures used for fermentation of extractant are listed below. The cultures were maintained on MGYP medium (Wickerham, 1951) (Appendix-I). The overnight grown yeast cultures in MGYP broth containing 30 x 10^6 cells ml^{-1} were used as inocula.

Table 1 : The yeast strains screened for bioethanol production

Sl. No.	Strains	Source
	Local isolates	
1.	BCY-101	Banana fruit
2.	BCY-102	Cashew fruit
3.	BCY-104	Curds
4.	BCY-105	Grapes
5.	BCY-106	Grapes
6.	BCY-107	Jack fruit
7.	BCY-108	Rain tree pods
	Standard isolates	
1.	*Saccharomyces cerevisiae* CFTRI 101	CFTRI-Mysore
2.	*Rhodotorula rubra* NCYC 195	CFTRI-Mysore
3.	*Candida shehate* NCIM 3500	NCIM-Pune
4.	*Saccharomyces cerevisiae* NCIM 3570	NCIM-Pune
5.	*Saccharomyces uvaram* NCIM 3576	NCIM-Pune
6.	*Candida wickerhami* NCIM 3463	NCIM-Pune
7.	*Kluveromyces marxianus* NCIM 3551	NCIM-Pune
8.	*Saccharomyces cerevisiae* NCIM 3288	NCIM-Pune
9.	*Kluveromyces marxianus* NCIM 3566	NCIM-Pune
10.	*Saccharomyces cerevisiae* NCIM 3090	NCIM-Pune
11.	*Pachysolen tannophilus* NCIM 3445	NCIM-Pune
12.	*Saccharomyces carlsbergensis* NCIM 3455	NCIM-Pune
13.	*Saccharomyces cerevisiae* NCIM 3580	NCIM-Pune

3.3.1 Fermentation

The acid hydrolysate of the pods of *S. saman* was adjusted to a pH of 4.5 using pellets of potassium hydroxide and inoculated with different yeast cultures and incubated under aerobic conditions for 24 hours. Anaerobic condition was then created by plugging with cork, making a provision for trapping carbon dioxide and incubated at room temperature for 7 days. The amount of ethanol produced and residual sugars left unfermented were estimated. The best performed strains (BCY-108, *C. wickerhami*, NCIM 3463 and *K. marxianus* NCIM 3551) were selected for further standardization.

3.4 EFFECT OF FERMENTATION TIME ON BIOETHANOL PRODUCTION

The efficient yeast cultures were tested to standardise the optimum period of fermentation for maximum ethanol production. The acid hydrolysate was inoculated @ one per cent yeast inoculum and was allowed for fermentation for 4, 5, 7 and 9 days. The residual sugar content and ethanol produced were analysed.

3.5 EFFECT OF pH ON BIOETHANOL PRODUCTION

The pH of the acid hydrolysate was adjusted to 3.5, 4.5, 5.5 and 6.5 with potassium hydroxide pellets. The efficient yeast cultures *viz.*, *C. wickerhami* NCIM 3463, *K. marxianus* NCIM 3551 and local isolate BCY-108 were inoculated and fermentation was continued upto 7 days as described earlier. The samples were analysed for ethanol and residual sugar content after fermentation.

3.6 EFFECT OF TEMPERATURE ON BIOETHANOL PRODUCTION

After determination of optimum pH which was 4.5, hydrolysate was inoculated with efficient yeast cultures @ 1.00 per cent and incubated at different temperatures *viz.*, 25, 30 and 37°C for 7 days. After complete fermentation, the samples were analysed for ethanol and residual sugar content.

3.7 STANDARDISATION OF INOCULUM SIZE FOR BIOETHANOL PRODUCTION

The pod extractant was further studied to know the effect of various levels of inoculum on ethanol production.

The pod extract (100 ml of pH 4.5) was inoculated with 1.0, 1.5 and 2.0 per cent of inoculum levels and kept for fermentation at 30°C for 7 days and samples were analysed for residual sugar and ethanol yield.

3.8 EFFECT OF NUTRIENT SUPPLEMENTATION ON BIOETHANOL PRODUCTION

The pod extractant was further studied to know the effect of supplementation of various nutrients and optimisation of their concentrations on ethanol yield. Mainly nitrogen, phosphorus and zinc sulphate were included in the study.

Ammonium sulphate was used to supply additional nitrogen, as it was proved elsewhere to be best source compared to other source. Phosphate was supplied in the form of orthophosphoric acid and sodium dihydrogen phosphate.

Zinc sulphate was supplemented as stimulant, since it is known to stimulate the alcohol dehydrogenase (ADH) enzyme which is responsible for alcohol production.

The concentrations of all the nutrients were used individually which are listed below. A control was also maintained without supplementation of any nutrients.

1. Ammonium sulphate - 0.1, 0.3 and 0.5 per cent

   (Anantha and Gunasekaran, 2000).
2. Orthophosphoric acid – 0.002, 0.004 and 0.006 per cent

   (Cuesta and Cornejo, 1988).
3. Sodium dihydrogen phosphate – 0.1, 0.3 and 0.5 per cent

   (Marakis and Marakis, 1996).
4. Zinc (as zinc sulphate) – 5, 10 and 15 mg L^{-1}

   (Sreenath and Jeffries, 2000).

The substrates supplemented with the above mentioned nutrients were inoculated individually with efficient yeast cultures @ 1.5 per cent inoculum and kept for fermentation for 5 days at 30°C. The samples were analysed for ethanol and residual sugar content.

3.9 CHEMICAL ANALYSIS

3.9.1 Estimation of reducing sugars

The reducing sugars were estimated by dinitro-salicylic acid (DNSA) method as described by Miller (1959).

3.9.1.1 Preparation of reagent

3.9.1.1.1 DNSA : 1 g of 3, 5,di-nitrosalicylic acid was dissolved in little amount of 2N NaOH and 30 g of sodium potassium tartrate was added and volume was made upto 100 ml with 2N NaOH.

3.9.1.2 Preparation of stock solution of glucose

Standard stock solution was prepared @ 1 mg ml^{-1} by dissolving 100 mg of D-glucose in distilled water and final volume was made upto 100 ml with distilled water.

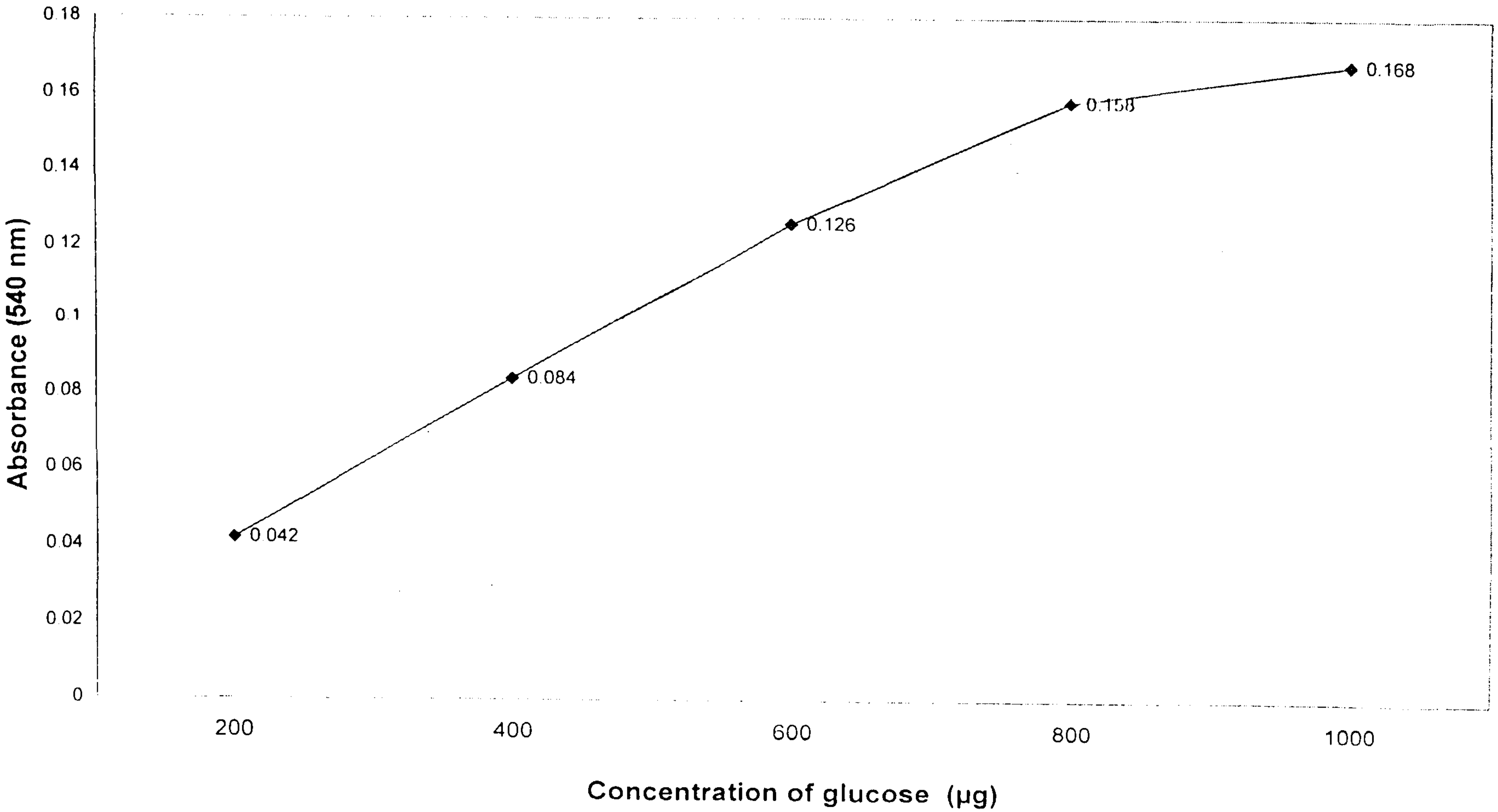


Fig. 4: Standard curve of glucose

3.9.1.3 Procedure

The representative samples of 0.1 ml from each treatment and replications was taken into thin walled boiling test tubes and 0.9 ml of distilled water was added. A reagent blank containing one ml of distilled water was also prepared. Similarly, standards were also included ranging from 100 µg to 1000 µg concentration of glucose. DNSA reagent @ 0.5 ml was added to each sample, mixed well and kept on boiling water bath for 5 minutes. The samples were cooled and final volume was made upto 25 ml using volumetric flask.

Absorbance in terms of optical density of the standard and the samples were read at 540 nm using Systronics UV spectrophotometer-117. The standard curve of glucose is presented in Fig.4.

3.9.2 Estimation of ethanol

The ethanol was estimated by colorimetric method as described by Caputi *et al.* (1968).

3.9.2.1 Preparation of reagent

3.9.2.1.1 Potassium dichromate ($K_2Cr_2O_7$) : 34 g of $K_2Cr_2O_7$ was dissolved in 500 ml of distilled water and 325 ml of sulphuric acid was added and volume was made up to 1000 ml with distilled water to give 0.23 N $K_2Cr_2O_7$.

3.9.2.2 Preparation of stock solution

Standard stock solution of 100 per cent pure analytical grade (containing 789 mg ml^{-1}) ethanol was prepared by dissolving 12.6 ml of ethanol in 100 ml distilled water, which resulted in 100 mg ml^{-1} of standard ethanol.

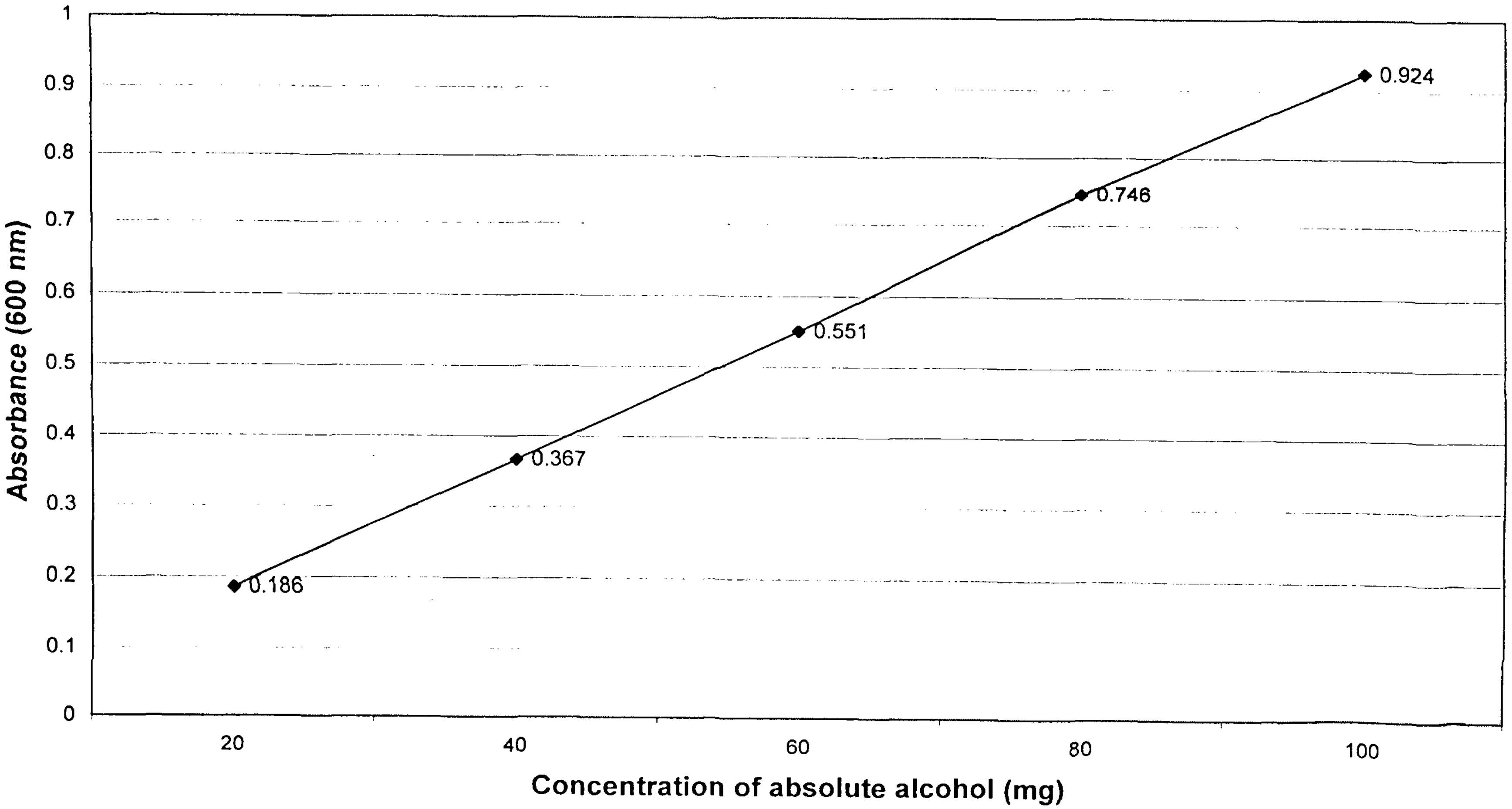


Fig. 5: Standard curve of ethanol

3.9.2.3 Procedure

One ml of the representative samples from each treatment was transferred to 250 ml round bottom distillation flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-75°C. The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagent which was kept at the receiving end. The distillate containing alcohol was collected till total volume of 45 ml was obtained. Similarly, standards (20-100 mg ethanol) were mixed with 25 ml of $K_2Cr_2O_7$ separately. The distillate of samples and the standards were heated in water bath at 60°C for 20 minutes and were cooled. The volume was made upto 50 ml with distilled water and the optical density was measured at 600 nm using Systronics spectrophotometer-117. The standard curve was plotted considering the concentration against absorbance (Fig.5).

3.10 ENUMERATION OF YEAST CELL COUNTS

3.10.1 Serial dilution and plate count technique

The sample from the pilot scale fermentor of 10 ml was serially diluted using 90 ml water blanks and suitable dilutions were plated under aseptic conditions and MGYP medium was poured. The plates were incubated after solidification at 35°C for 24 h. The colony forming units (CFU) were counted.

3.11 PILOT SCALE PRODUCTION OF ETHANOL

From the previous experiments, BCY-108 yeast strain was found to be efficient in conversion of the hydrolysate to ethanol. Hence, this strain was used for pilot scale fermentation of ethanol. The optimum parameters obtained from the previous studies were adopted in this trial. A fermentor of 3L was designed for the pilot scale study in which 1.5 L of the hydrolysate was fermented by following the procedure described before. The performance was evaluated in terms of ethanol yield and residual sugars. The population density was also observed at intervals.

3.11.1 Optimum fermentation parameters followed for pilot scale production of ethanol

Parameters

Fermentation time : 5 days

pH : 4.5

Temperature : 30°C

Inoculum size : 1.5 per cent (30 x 10^6 cells ml^{-1})

Nutrient supplementatoin : $ZnSO_4$ @ 10 mg L^{-1}.

3.12 STATISTICAL ANALYSIS

The results obtained were analysed statistically using completely randomised design as described by Panse and Sukhatme (1985).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Ethanol being a biosolar fuel has gained importance of late in the transport sector as it can be blended upto 20 per cent with diesel or petrol. At present, ethanol is produced from molasses which is a byproduct of sugar industries. Most of the distilleries operate at high cost as the cost of molasses is very high. Hence, it is absolutely necessary to search for alternate sources for ethanol production. So far, common crops recognised are sugarcane, sweet sorghum, beet and potato. Most of such products involve multistep processing for releasing the readily available sugars which might increase the total cost. But the pods of tropical tree *Samanea saman* (Jacq.) Merr. contain considerable amount of nutrients which can form an ideal medium for ethanol production. Therefore, the substrate was evaluated for ethanol production using various means for releasing sugars and optimising various parameters including selection of yeast strains, pH, temperature, fermentation time, inoculum size and nutrient supplementation etc. The results are being presented in this chapter.

4.1 CHEMICAL COMPOSITION OF *Samanea saman*

It was essential to know the natural constituents of deseeded pods of *S. saman*. Hence, proximate analysis was carried out. The results are presented in Table 2. The pulp portion of the pods contain total sugar (460 mg g^{-1}), reducing sugar (210 mg g^{-1}), organic carbon (40.6%), nitrogen (1.05%), protein (6.56%), tannin (2.95%), phenol (3.875 mg g^{-1}), total solids (80%), moisture (20%), pH (4.56) and C:N ratio (38.66).

4.2 EXTRACTANT (FILTRATE)

Initially to know the volume of extract and maximum sugars, 1 kg of deseeded pods of *S. saman* was dissolved in 6L of water (1:6 w/v) and

Table 2 : Biochemical properties of raw deseeded pods of *S. saman*

Sl.No.	Parameters	Content
1.	Total solids	80.0%
2.	Moisture	20.0%
3.	Organic carbon	40.6%
4.	Phenols	3.875 mg g^{-1}
5.	Total sugar	460 mg g^{-1}
6.	Nitrogen	1.05%
7.	Protein	6.56%
8.	Reducing sugar	210 mg g^{-1}
9.	Tannin	2.95%
10.	pH	4.56
11.	C:N ratio	38.66

filtered twice with double layered muslin cloth and finally once with ordinary filter paper. The ratio of 1:6 (w/v) was optimum which produced 3.4 L of filtrate.

4.3 PRETREATMENT OF SUBSTRATE FOR MAXIMUM EXTRACTION OF REDUCING SUGAR FROM DESEEDED PODS BY AQUEOUS EXTRACTION

In general, aqueous extraction had positive correlation with the release of reducing sugars in deseeded pods of *S. saman* and different incubation periods, which ranged from 15.463 to 19.770 g L^{-1} (Table 3). The reducing sugar content in deseeded pods of *S. saman* increased significantly with increase in incubation periods upto 8h, beyond which, increase in reducing sugar content was found to be non-significant and they were significantly superior as compared to other incubation periods (Table 3).

4.4 EFFECT OF INCUBATION TEMPERATURES AND INCUBATION PERIODS ON REDUCING SUGAR FROM THE PODS

The reducing sugar content increased with increase in temperature significantly, which ranged from 17.564 to 23.559 g L^{-1} when incubated for 10 hours. The highest reducing sugar content in deseeded pods of *S. saman* recorded was 23.559 g L^{-1} at 100°C, followed by 80°C which showed 23.504 g L^{-1}. At ambient temperature and 50°C recorded 17.564 and 18.276 g L^{-1} respectively (Table 4) during 10 hours of incubation. However, the reducing sugar increased significantly upto 8 hours, beyond which increase in reducing sugar was not significant. At 8, 9 and 10 hours, sugar recorded was 22.958, 22.966 and 22.977 g L^{-1}, respectively, which were significant as compared to other periods of

Table 3 : Influence of incubation periods on reducing sugars by aqueous extraction of deseeded pods of *S. saman*

Sl. No.	Incubation period (h)	Reducing sugar ($g\ L^{-1}$)
1.	1.	15.463
2.	2.	15.660
3.	3.	15.967
4.	4.	16.277
5.	5.	16.883
6.	6.	17.527
7.	7.	18.613
8.	8.	19.723
9.	9.	19.757
10.	10.	19.770
	SEm±	0.0109
	CD at 1%	0.045

Table 4:Effect of interaction of temperature and incubation periods on reducing sugar from deseeded pods of *S. saman*

Sl. No.	Incubation period (h)	Reducing sugars (g L^{-1})				
		Temperature (°C)				
		Room temperature (28±1°C)	50	80	100	Mean
1.	1	15.463	16.187	20.503	20.553	18.177
2.	2	15.660	16.367	20.867	20.917	18.453
3.	3	15.967	16.783	21.843	21.893	19.122
4.	4	16.277	17.243	22.483	22.550	20.638
5.	5	16.883	17.870	22.883	22.933	20.143
6.	6	17.527	18.547	23.837	23.887	21.877
7.	7	18.613	19.370	24.737	24.787	22.949
8.	8	19.723	20.120	25.953	26.037	22.958
9.	9	19.757	20.123	25.967	26.017	22.966
10.	10	19.770	20.147	25.970	26.020	22.977
	Mean	17.564	18.276	23.504	23.559	
	Source	SEm±	CD at 1%			
	Temperature (A)	0.0033	0.0123			
	Incubation period (B)	0.0053	0.0197			
	A X B	0.0106	0.0395			

incubation (Table 4). Thus an incubation period of 8-10 h is optimum for maximum release of reducing sugars.

The interaction effect between temperature and incubation period was significant on reducing sugar content of deseeded pods of *S. saman.* The highest reducing sugar content (26.037 g L^{-1}) was recorded at 100°C at 8 hours of incubation period and further release of sugar almost remained constant upto 10 h of incubation. Thus release of sugar was significant at 100°C for 8 h of incubation as compared to other temperatures and incubation periods.

4.5 EFFECT OF BIOLOGICAL PRETREATMENT ON REDUCING SUGARS AND PER CENT LOSS IN WEIGHT

The deseeded pods of *S. saman* inoculated with different fungal cultures had highest reducing sugar content and per cent loss in weight as compared to uninoculated control (Table 5).

Among fungal cultures, substrate inoculated with *A. foetidus* (NCIM 515) recorded highest reducing sugar and per cent loss in weight (24.26 g L^{-1} and 48.32 per cent respectively) followed by *A. niger* (NCIM 616), which recorded 20.66 g L^{-1} and 44.00 per cent loss in weight irrespect of the conditions.

The un-sterilised substrate showed significant release of sugars (24.11 g L^{-1}) as compared to sterilized conditions (12.61 g L^{-1}) irrespective of either fungal cultures.

In sterile conditions also, *A. foetidus* (NCIM 515) showed maximum release of sugars and loss of weight, but were significantly lower as compared to unsterile conditions. Similarly, *A. niger* (NCIM 616) also showed similar trend as compared to control.

Table 5 : Effect of biological pretreatment on reducing sugars and per cent loss in weight of deseeded pods of *S. saman*

Sl. No.	Treatments	Reducing sugars (g L^{-1})			Loss in weight (%)		
		Unsterilized	Sterilized	Mean	Unsterilized	Sterilized	Mean
1.	Control	19.13	7.60	13.37	38.00	20.00	29.00
2.	*Aspergillus. foetidus* (NCIM 515)	31.51	17.01	24.26	63.33	33.31	48.32
3.	*A awamori* (IARI)	23.22	12.49	17.86	46.66	24.00	35.33
4.	*A. niger* (NCIM 616)	26.97	14.36	20.66	60.66	27.33	44.00
5.	*A. niger* (Local isolate)	19.72	11.58	15.65	40.00	22.00	31.00
	Mean	24.11	12.61		50.13	25.32	
	Source	SEm±	CD at 1%		SEm±	CD at 1%	
	Culture (A)	0.0073	0.02936		0.2982	0.1999	
	Conditions (B)	0.0046	0.019		0.1886	0.7587	
	A X B	0.0103	0.0413		0.4216	1.696	

4.6 EFFECT OF ALKALI-PRETREATMENT AND INCUBATION PERIODS ON REDUCING SUGAR CONTENT OF DESEEDED PODS OF *S. saman*

The reducing sugar content in deseeded pods of *S. saman* increased significantly as the concentrations of alkali increased. The highest reducing sugar content was recorded at 1.0 and 1.5 per cent alkali concentrations, which accounts to 27.793 and 27.824 g L^{-1}, respectively and they were significantly superior to 0.5 per cent alkali treatment and control which recorded 25.271 and 17.564 g L^{-1} respectively (Table 6).

Similarly, the reducing sugar content also increased significantly with increase in incubation periods (1 to 10 h), which ranged from 22.543 and 26.516 g L^{-1} (Table 6). However, reducing sugar content increased significantly upto 8 hours, beyond which increase in reducing sugar content was non-significant. However, the reducing sugar at 8, 9 and 10 hrs of incubation was significantly higher as compared to other periods of incubation (Table 6).

The interaction effect between alkali concentration and incubation period was significant on reducing sugar content of deseeded pods of *S. saman*. The highest reducing sugar content was recorded at 1.5 and 1.0 per cent alkali concentrations at 8, 9 and 10 hours of incubation, which was 29.770, 29.780 and 29.793, and 29.740, 29.750 and 29.770 g L^{-1} respectively. However, the interaction effect did not differ significantly in treatment combinations receiving 1.5 and 1.0 per cent alkali concentrations at 8, 9 and 10 hours of incubation periods. Thus, exposure of pods to 1 per cent alkali pretreatment for 8 h was found to be optimum for maximum release of reducing sugars.

Table 6 : Effect of alkali pretreatment and incubation periods on the extraction of reducing sugar content from deseeded pods of *S. saman*

Sl. No.	Incubation period (h)	Reducing sugars (g L^{-1})				
		Sodium hydroxide (%)				
		Control	0.5	1.0	1.5	Mean
1.	1	15.463	23.537	25.570	25.600	22.543
2.	2	15.660	23.730	25.880	25.910	22.795
3.	3	15.967	23.930	26.253	26.283	23.108
4.	4	16.277	24.530	26.747	26.777	23.583
5.	5	16.883	25.337	27.407	27.437	24.266
6.	6	17.527	25.493	27.960	27.987	24.742
7.	7	18.613	26.053	28.857	28.903	25.607
8.	8	19.723	26.663	29.740	29.770	26.474
9.	9	19.757	26.710	29.750	29.780	26.499
10.	10	19.770	26.730	29.770	29.793	26.516
	Mean	17.564	25.271	27.793	27.824	
	Source	SEm±	CD at 1%			
	Alkali (A)	0.0167	0.06229			
	Incubation period (B)	0.0263	0.09810			
	A X B	0.0527	0.1965			

4.7 EFFECT OF ACID CONCENTRATION AND INCUBATION PERIODS ON REDUCING SUGAR CONTENT IN DESEEDED PODS OF *S. saman*

Similar to alkali pretreatment, acid pretreatment also showed increase in reducing sugars as the concentration of acid increased along with increase in periods of incubation. The results are being presented in Table 7.

The reducing sugar content of deseeded pods of *S. saman* was found to be significantly increased due to different acid treatments which ranged from 17.564 to 33.067 g L^{-1}. Reducing sugar content increased significantly with increase in acid concentration upto 1.50 per cent (33.067 g L^{-1}), but it did not differ significantly with 1.0 per cent acid concentration (32.936 g L^{-1}). Both these treatments were significantly superior as compared to other treatments and control (17.564 g L^{-1}).

Similarly, the reducing sugar content also increased significantly with increase in incubation period from one to ten hours, which ranged from 24.332 g to 28.547 g L^{-1}. However, increase in reducing sugar content was significant upto 8 hours beyond which, increase was not significant. These treatments were significantly superior as compared to other incubation periods (Table 7).

The interaction effect between acid concentrations and incubation periods was significant on reducing sugar content of deseeded pods of *S. saman*. The highest reducing sugar content was recorded in both 1.0 and 1.5 per cent acid concentrations at 9th and 10th hour of incubation period (Fig.6). At 1 per cent acid, sugar was 35.203 g L^{-1} and 35.243 g L^{-1} at 1.5 per cent acid pretreatment for 9 h of incubation, which did not differ

Table 7 : Effect of acid pretreatment and incubation periods on the extraction of reducing sugar from deseeded pods of *S. saman*

Sl. No.	Incubation period (h)	Reducing sugars (g L^{-1})					
		Sulphuric acid (%)					
		Control	0.1	0.5	1.0	1.5	Mean
1.	1	15.463	20.510	24.570	30.513	30.553	24.322
2.	2	15.660	20.700	24.877	30.863	30.903	24.601
3.	3	15.967	20.900	25.260	31.357	31.397	24.976
4.	4	16.277	21.500	25.753	31.967	32.007	25.501
5.	5	16.883	21.973	26.400	32.700	32.740	26.139
6.	6	17.527	22.463	26.993	33.307	33.347	26.727
7.	7	18.613	23.023	27.853	33.987	34.027	27.501
8.	8	19.723	23.633	28.740	34.237	35.197	28.306
9.	9	19.757	23.680	28.750	35.203	35.243	28.527
10.	10	19.770	23.700	28.767	35.233	35.260	28.547
	Mean	17.564	22.208	26.796	32.936	33.067	
	Source	SEm±	CD at 1%				
	Acid (A)	0.0445	0.165				
	Incubation period (B)	0.0630	0.234				
	A X B	0.1409	0.5232				

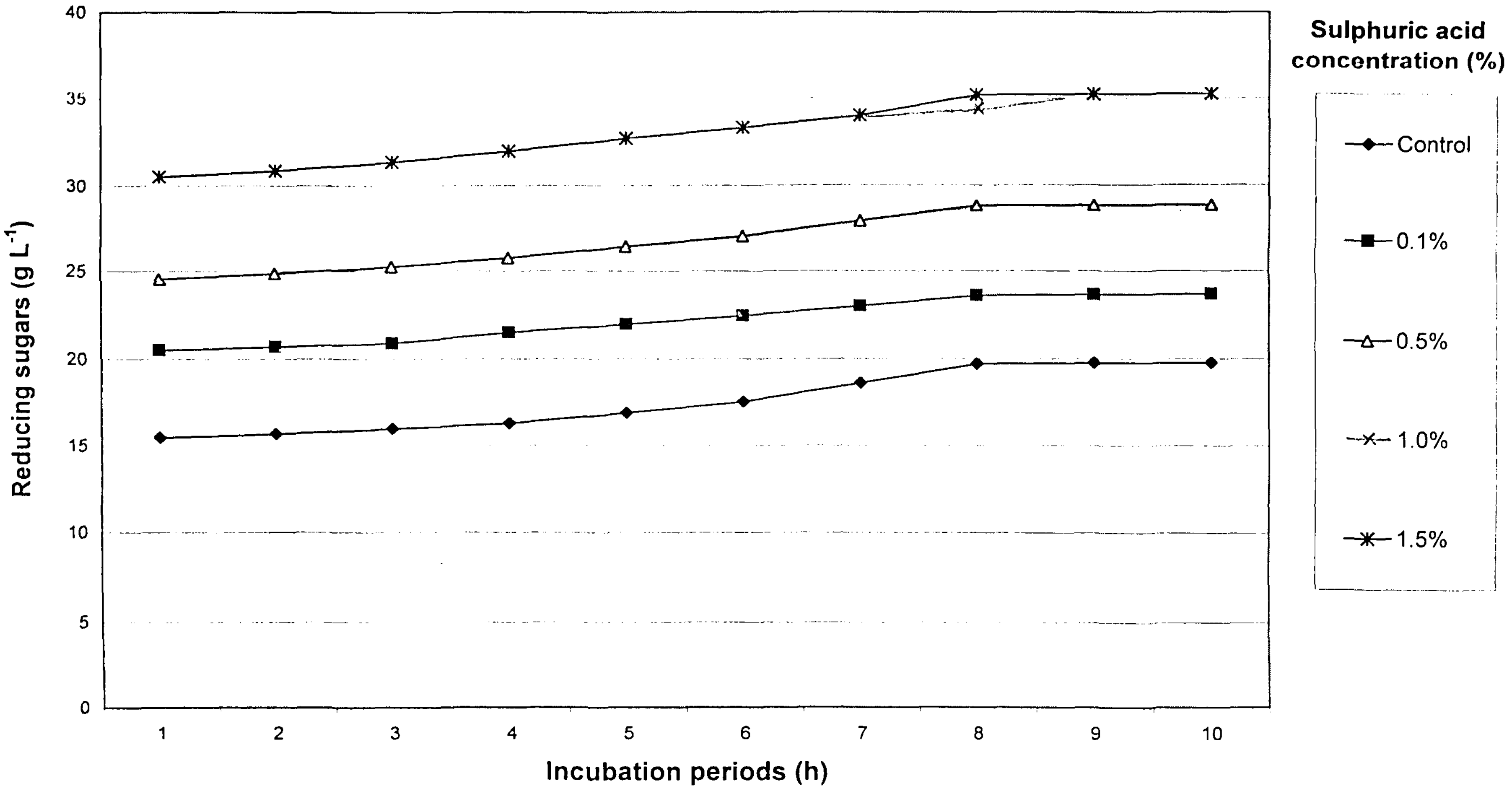


Fig. 6: Effect of acid pre treatment and incubation periods on the extraction of reducing sugar from deseeded pods of *S. saman*

significantly at 10th hour of incubation but was significantly superior to other treatment combinations. The optimum conditions for maximum extraction of sugar was 1.5 per cent acid for 8-10 h of incubation which was followed for all other experiments.

4.8 SCREENING OF EFFICIENT YEAST CULTURES FOR ETHANOL YIELD

In general, the acid hydrolysate supported the growth of yeast cultures and production of ethanol (Table 8).

The yeast cultures differed significantly in their ability to ferment acid hydrolysate of deseeded pods of *S. saman*. The ethanol yield increased significantly due to inoculation of different yeast cultures, which ranged from 12.207 to 20.257 g L^{-1} as compared to control (3.043 g L^{-1}). Among yeast cultures, BCY-108, *Candida wickerhami* NCIM 3463 and *Kluveromyces marxianus* NCIM 3551 had significantly higher ethanol yield (20.257, 19.813 and 19.630 g L^{-1}, respectively). These showed significance with each other as compared to other standard cultures and uninoculated control. Similarly, residual sugar content also decreased significantly in fermented acid hydrolysate of deseeded pods of *S. saman* inoculated with different yeast cultures. Significant decrease in residual sugar content was noticed when inoculated with BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 (3.567, 3.700 and 3.833 g L^{-1} respectively) as compared to other yeast cultures (Table 8 and Fig.7). Therefore, BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 yeast cultures were used for further standardisation of various parameters to obtain maximum ethanol.

Table 8 : Screening of efficient yeast strains for ethanol yield from acid hydrolysate of deseeded pods of *Samanea saman*

Sl. No.	Yeast cultures	Ethanol yield (g L^{-1})	Residual sugars (g L^{-1})
1.	Uninoculated control	3.043	8.767
	Local isolates		
2.	BCY-101	17.550	4.467
3.	BCY-102	17.983	4.300
4.	BCY-104	17.657	4.483
5.	BCY-105	18.010	4.367
6.	BCY-106	12.207	6.100
7.	BCY-107	18.407	4.177
8.	BCY-108	20.257	3.567
	Standard cultures		
9.	*Saccharomyces cerevisiae* CFTRI 101	19.330	4.047
10.	*Rhodotorula rubra* NCYC 195	17.317	4.583
11.	*Candida shehate* NCIM 3500	17.043	4.600
12.	*Saccharomyces cerevisiae* NCIM 3570	16.937	4.833
13.	*Saccharomyces uvaram* NCIM 3576	18.540	4.133
14.	*Candida wickerhami* NCIM 3463	19.813	3.700
15.	*Kluveromyces marxianus* NCIM 3551	19.630	3.833
16.	*Saccharomyces cerevisiae* NCIM 3288	17.837	4.393
17.	*Kluveromyces marxianus* NCIM 3566	18.390	4.193
18.	*Saccharomyces cerevisiae* NCIM 3090	14.823	5.467
19.	*Pachysolen tannophilus* NCIM 3445	18.600	4.167
20.	*Saccharomyces carlsbergensis* NCIM 3455	17.967	4.200
21.	*Saccharomyces cerevisiae* NCIM 3580	17.993	4.250
	SEm±	0.020	0.023
	CD at 1%	0.0804	0.093

Initial reducing sugar of acid hydrolysate = 35.26 g L^{-1}.

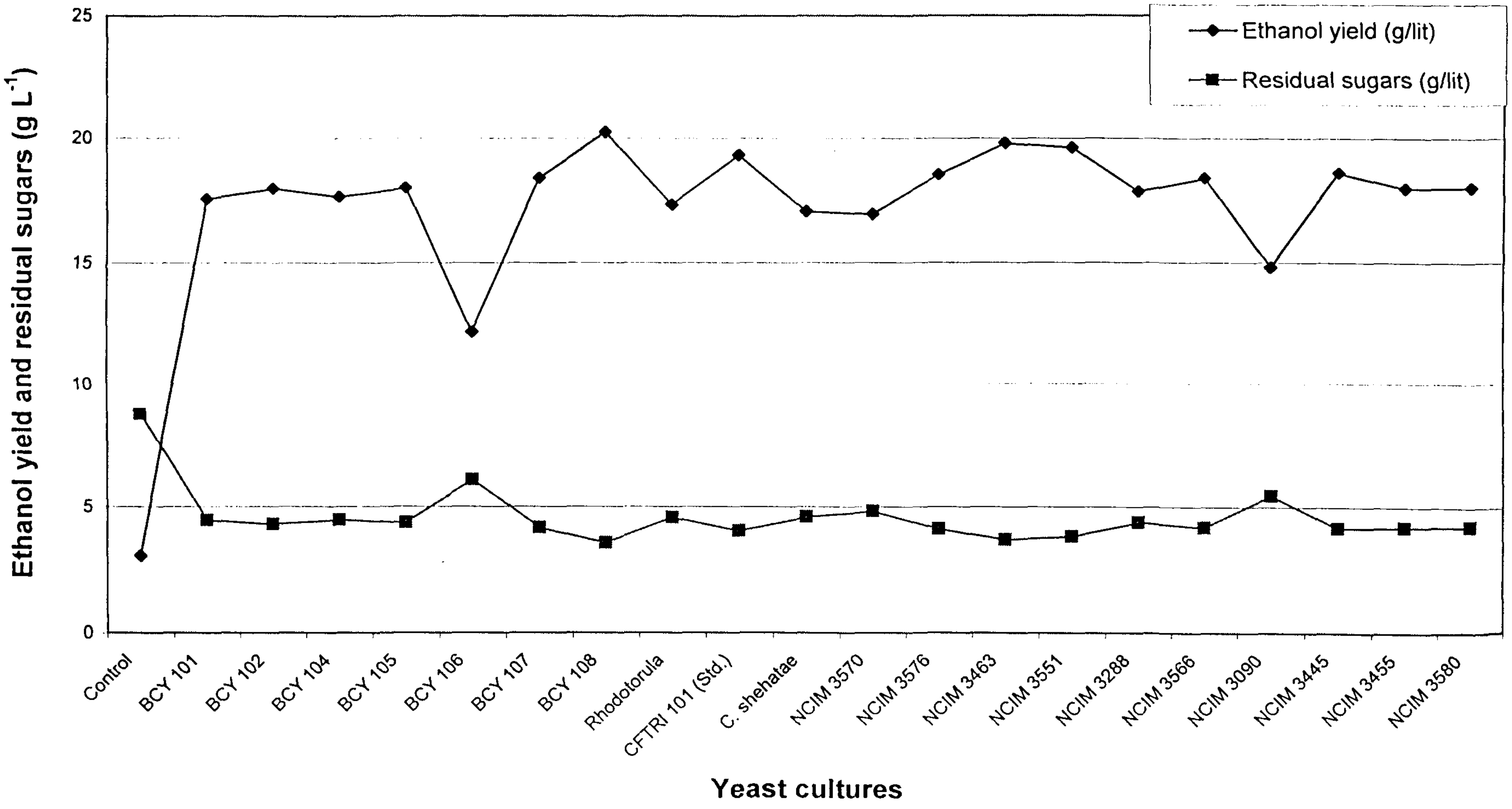


Fig. 7: Screening of efficient yeast strains for ethanol yield from acid hydrolysate of deseeded pods of *S.saman*

4.9 EFFECT OF DIFFERENT FERMENTATION PERIODS ON ETHANOL YIELD AND RESIDUAL SUGAR CONTENT IN DESEEDED PODS OF *S. saman*

The ethanol yield in deseeded pods of *S. saman* was found to increase due to inoculation of the selected efficient yeast cultures at different periods of fermentation. Accordingly the residual sugar content also decreased, as observed in Table 9.

The ethanol yield increased significantly in the acid hydrolysate of deseeded pods of *S. saman*, when inoculated with yeast cultures *viz.*, BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 (9.582, 9.512 and 9.377 g L^{-1} respectively) over respective control (Table 9). However, the three strains were on par with each other. The uninoculated control exhibited 4.267 g L^{-1} of ethanol during 9 days of fermentation.

The ethanol yield increased significantly with increase in fermentation period from 4 to 7 days. Further increase in ethanol yield did not differ significantly between 7th and 9th days (20.002 and 20.010 g L^{-1} respectively). The ethanol yield recorded on 7th and 9th day was significantly superior as compared to 4th and 5th day of fermentation (Table 9).

Similarly, the residual sugar content also decreased significantly in the acid hydrolysate of pods of *S. saman* due to inoculation of efficient yeast cultures *viz.*, BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551, which recorded 6.921, 6.972 and 7.060 g L^{-1} respectively (Table 9).

Table 9 : Effect of fermentation periods on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	Fermentation period (days)	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
		BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	Control	1.633	1.633	1.633	1.633	9.676	9.767	9.767	9.767
2.	4	10.537	10.143	9.913	10.198	6.723	6.847	6.983	6.851
3.	Control	1.833	1.833	1.833	1.833	9.200	9.200	9.220	9.200
4.	5	15.200	15.053	14.743	14.999	5.247	5.377	5.530	5.384
5.	Control	2.983	2.983	2.983	2.983	8.880	8.880	8.880	8.880
6.	7	20.260	20.013	19.733	20.002	3.663	3.703	3.943	3.770
7.	Control	3.933	4.433	4.433	4.267	8.233	8.233	8.233	8.233
8.	9	20.277	20.007	19.747	20.010	3.653	3.770	3.937	3.787
	Mean	9.582	9.512	9.377		6.921	6.972	7.06	
	Source	SEm±	CD at 1%			SEm±	CD at 1%		
	Fermentation period (A)	0.0763	0.2889			0.2924	1.1072		
	Cultures (B)	0.0467	0.1768			0.1790	0.6778		
	A X B	0.1322	0.5006			0.5064	1.9175		

The residual sugar content also decreased due to extended fermentation period from 4th to 9th day which ranged from 6.851 to 3.787 g L^{-1}. However, it decreased significantly upto 7th day of fermentation (3.770 g L^{-1}) beyond which decrease in residual sugar content did not differ significantly. In control, the residual sugars remained almost constant (8.233 g L^{-1}) during 9 days of fermentation.

The interaction effect was non-significant between fermentation time and yeast cultures on ethanol yield and residual sugar content in the acid hydrolysate of deseeded pods of *S. saman*. The ethanol yield was significant at 7 days of fermentation with BCY-108 strain (20.260 g L^{-1}), but it did not differ significantly on 9th day (20.277 g L^{-1}) of fermentation. The residual sugar content also decreased significantly.

4.10 EFFECT OF DIFFERENT pH LEVELS ON ETHANOL YIELD AND RESIDUAL SUGAR IN THE ACID HYDROLYSATE OF DESEEDED PODS OF S. *saman*

In general the ethanol yield was affected by pH levels as indicated in Table 10.

The ethanol yield was 13.591 and 19.953 g L^{-1} at pH levels of 3.5 and 4.5, respectively which was significant. Beyond pH 4.5 ethanol yield decreased significantly. The highest ethanol yield was recorded in acid hydrolysate at pH 4.5 inoculated with BCY-108 (20.257 g L^{-1}) which was significant as compared to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551. In control also, higher pH inhibited ethanol production with a corresponding increase in residual sugars.

Accordingly, the residual sugar content also decreased significantly at pH 4.5 which was optimum. As the pH increased, there was increase

Table 10 : Effect of different levels of pH on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	PH	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
		BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	Control	1.053	1.053	1.053	1.053	10.367	10.367	10.367	10.367
2.	3.5	13.800	13.650	13.323	13.591	5.810	5.927	5.983	5.907
3.	Control	3.597	3.597	3.597	3.597	7.883	7.883	7.883	7.883
4.	4.5	20.257	19.857	19.747	19.953	3.703	3.893	4.010	3.869
5.	Control	2.950	2.950	2.950	2.950	8.943	8.943	8.943	8.943
6.	5.5	17.340	17.153	17.013	17.169	4.613	4.753	4.837	4.734
7.	Control	2.047	2.047	2.047	2.047	9.310	9.310	9.310	9.310
8.	6.5	16.203	15.957	14.517	15.559	4.920	5.200	5.530	5.217
	Mean	9.656	9.533	9.281		6.944	7.035	7.108	
	Source	SEm±	CD at 1%			SEm±	CD at 1%		
	pH (A)	0.0171	0.0647			0.0283	0.1071		
	Cultures (B)	0.0105	0.03976			0.0173	0.0655		
	A X B	0.0296	0.1120			0.0490	0.1855		

in the residual sugar which was 4.734 g L^{-1} at pH 5.5 and 5.217 g L^{-1} at pH 6.5, indicating their inhibitory effect on fermentation process (Table 10).

4.11 EFFECT OF DIFFERENT TEMPERATURE ON ETHANOL YIELD AND RESIDUAL SUGAR CONTENT IN THE ACID HYDROLYSATE OF DESEEDED PODS OF S. *saman*

Among cultures, highest ethanol yield was recorded in BCY-108 strain as compared to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 (Table 11).

The ethanol yield was influenced by various temperatures. It increased significantly upto 30°C, beyond which, it decreased significantly. The highest ethanol yield was noticed at 30°C, which was 20.004 g L^{-1} (Table 11).

Similarly, residual sugar content decreased significantly in BCY-108 (4.818 g L^{-1}) as compared to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551, which recorded 5.104 and 5.346 g L^{-1}, respectively (Table 11).

Among temperatures, the residual sugar content decreased significantly at 30°C which recorded 3.952 g L^{-1} as compared to 25 and 37°C which accounts to 6.419 and 4.897 g L^{-1}.

However, strain BCY-108 showed significantly decrease in residual sugars (3.713 g L^{-1}) at 30°C as compared to other treatment combinations .The higher temperature affected the fermentation process and there by the residual sugar content has increased.

Table 11 : Effect of incubation temperature on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	Incubation temperature (°C)	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
		BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	25	12.953	11.457	10.513	11.641	6.127	6.417	6.713	6.419
2.	30±1 (room temperature)	20.317	19.963	19.733	20.004	3.713	3.980	4.163	3.952
3.	37	17.303	16.230	15.687	16.407	4.613	4.917	5.160	4.897
	Mean	16.858	15.883	15.311		4.818	5.104	5.346	
	Source	SEm±	CD at 1%			SEm±	CD at 1%		
	Temperature (A)	0.0064	0.0262			0.0051	0.0210		
	Cultures (B)	0.0064	0.0262			0.0051	0.0210		
	A X B	0.011	0.0451			0.0089	0.0365		

4.12 EFFECT OF DIFFERENT INOCULUM SIZE ON ETHANOL YIELD AND RESIDUAL SUGAR CONTENT IN THE ACID HYDROLYSATE OF DESEEDED PODS OF *S. saman*

In general, ethanol yield and residual sugar content in the acid hydrolysate of deseeded pods of *S. saman* were significantly affected by inoculum size.

The inoculum @ 30 x 10^6 cfu ml^{-1} was inoculated at 1.00, 1.5 and 2.0 ml for optimising the level for maximum ethanol production.

Among yeast cultures, BCY-108 recorded significantly higher ethanol yield (23.407 g L^{-1}), and was superior to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551, which accounts to 22.770 and 22.163 g L^{-1}, respectively (Table 12).

The residual sugar content also decreased significantly when acid hydrolysate was inoculated with BCY-108 (2.831 g L^{-1}) as compared to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551, which showed 3.036 and 3.282 g L^{-1}, respectively (Table 12).

Inoculum size showed significant effect on ethanol yield, which increased significantly with increase in level of inoculum upto 1.5 per cent. Further increase in ethanol yield did not differ significantly. But, the highest ethanol yield of 24.131 and 24.154 g L^{-1} was observed when provided with 1.5 and 2.0 per cent inoculum, respectively. From the visual observations the production of carbon dioxide was seized at 5 days of incubation in case of 1.5 per cent inoculum size, indicating the completion of fermentation. Hence the optimum of 1.5 per cent inoculum with incubation for 5 days was used in further experiments, since 1.0 per cent inoculum size showed 7 days of fermentation.

Table 12 : Effect of inoculum size on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	**Inoculum size (%)**	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
		BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	1.0	20.520	19.937	19.707	20.054	3.720	4.130	4.230	4.027
2.	1.5	24.837	24.177	23.380	24.131	2.403	2.497	2.820	2.573
3.	2.0	24.863	24.197	23.403	24.154	2.370	2.480	2.797	2.549
	Mean	23.407	22.770	22.163		2.831	3.036	3.282	
	Source	SEm±	CD at 1%			SEm±	CD at 1%		
	Inoculum size (A)	0.0121	0.0496			0.0124	0.0508		
	Cultures (B)	0.0121	0.0496			0.0124	0.0508		
	A X B	0.0209	0.0856			0.0214	0.0876		

Uninoculated control : Ethanol yield = 3.043 g L^{-1}
Residual sugar = 8.767 g L^{-1}.

While the residual sugar content also decreased significantly at 1.5 per cent inoculum size (2.573 g L^{-1}). Further, decrease in residual sugar content was non-significant at 2.0 per cent inoculum size (2.549 g L^{-1}) (Table 12).

The interaction effect between yeast cultures and inoculum size was significant, on ethanol yield and residual sugar content (Table 12). Though ethanol yield was maximum (24.863 g L^{-1}) at 2.0 per cent inoculum exhibited by BCY-108 strain, it was non significant with 1.5 per cent inoculum (24.837 g L^{-1}). The other two cultures also showed similar trend. Least ethanol was produced at 1.0 per cent inoculum in all the cultures tested and also reducing sugars accumulated significantly. BCY-108 was superior and inoculum size of 1.5 per cent was optimum for maximum ethanol production.

4.13 EFFECT OF NUTRIENT SUPPLEMENTATION AT DIFFERENT CONCENTRATIONS ON ETHANOL YIELD AND RESIDUAL SUGAR CONTENT IN THE ACID HYDROLYSATE OF DESEEDED PODS OF *S. saman*

The hydrolysate was supplemented with nutrient for further enhancing the ethanol yield. The composition of the pods indicates 40.6 per cent of organic carbon and 1.05 per cent of nitrogen, accounting to 38.66 of C:N ratio which is quite high for efficient microbial activity. But, supplementing with N and P sources and $ZnSO_4$ as stimulant, could further enhance ethanol yield. With this objective, a study was conducted to know their effect on ethanol yield. The results are being presented in Table 13a and 13b.

Among yeast cultures BCY-108 recorded highest ethanol yield (25.176 g L^{-1}), as compared to *C. wickerhami* NCIM 3463 and

K. marxianus NCIM 3551, which recorded 24.664 and 23.857 g L^{-1} in the acid hydrolysate supplemented with orthophosphoric acid. While the residual sugar content also decreased significantly when inoculated BCY-108 which recorded 2.396 g L^{-1} and was superior to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 which recorded 2.548 and 2.693 g L^{-1}, respectively (Table 13a).

The ethanol yield increased significantly when acid hydrolysate was supplemented with orthophosphoric acid upto 0.004 per cent (27.228 g L^{-1}), beyond which it decreased significantly. While the residual sugar content also decreased significantly at 0.004 per cent which recorded 2.061 g L^{-1}.

Another P source, sodium dihydrogen orthophosphate (NaH_2PO_4) was also studied. The ethanol yield was maximum at 0.1 per cent NaH_2PO_4 (19.809 g L^{-1}) followed by 0.3 per cent, beyond which it decreased significantly. While residual sugar content also decreased significantly at 0.1 per cent, which was 3.898 g L^{-1}. Further increase in the NaH_2PO_4, there was increase in residual sugars.

When Ammonium sulphate [$(NH_4)_2 SO_4$] was supplemented, highest ethanol yield was recorded at 0.3 per cent of $(NH_4)_2 SO_4$ which was 26.966 g L^{-1}. The residual reducing sugars in 0.3 per cent supplementation was 1.742 g L^{-1} which is significantly less as compared to that of 0.1 and 0.5 per cent of $(NH_4)_2SO_4$.

Similarly, the acid hydrolysate when supplemented with zinc sulphate ($ZnSO_4$), the ethanol yield was found to be increased significantly upto 10 mg L^{-1} (29.849 g L^{-1}), beyond which the ethanol yield decreased significantly. The residual sugar content also decreased

Table 13a : Effect of nutrient supplementation on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	Nutrients	Concentrations	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
			BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	Orthophosphoric acid (H_3PO_4) (%)	Control	24.800	24.137	23.340	24.092	2.437	2.537	2.860	2.611
		0.002	24.897	24.323	23.470	24.230	2.340	2.393	2.513	2.416
		0.004	27.763	27.547	26.373	27.228	1.877	2.140	2.167	2.061
		0.006	23.243	22.650	22.243	22.712	2.930	3.120	3.230	3.093
		Mean	25.176	24.664	23.857	24.566	2.396	2.548	2.693	2.545
2.	Sodium dihydrogen orthophosphate (NaH_2PO_4) (%)	Control	24.800	24.137	23.340	24.092	2.437	2.537	2.860	2.611
		0.1	20.327	19.740	19.360	19.809	3.717	3.960	4.017	3.898
		0.3	17.753	16.670	15.970	16.798	4.623	4.920	5.073	4.872
		0.5	16.373	15.727	15.243	15.781	5.047	5.207	5.303	5.186
		Mean	19.813	19.068	18.478	19.120	3.956	4.156	4.313	4.142

Table 13b : Effect of nutrient supplementation on ethanol yield and residual sugar content in the extract of deseeded pods of *S. saman*

Sl. No.	Nutrients	Concentrations	Ethanol yield (g L^{-1})				Residual sugar (g L^{-1})			
			BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean	BCY-108	*C. wickerhami* NCIM 3463	*K. marxianus* NCIM 3551	Mean
1.	Ammonium sulphate $(NH_4)_2SO_4$(%)	Control	24.800	24.137	23.340	24.092	2.437	2.537	2.860	2.611
		0.1	25.330	25.103	24.767	25.067	2.410	2.613	2.820	2.614
		0.3	27.963	26.760	26.173	26.966	1.467	1.587	2.173	1.742
		0.5	23.427	22.607	22.273	22.769	2.837	2.133	3.253	2.741
		Mean	25.380	24.652	24.138	24.723	2.288	2.218	2.777	2.427
2.	Zinc sulphate $(ZnSO_4)$ (mg L^{-1})	Control	24.800	24.137	23.340	24.092	2.437	2.537	2.860	2.611
		5	25.937	25.203	24.753	25.298	2.233	2.357	2.513	2.368
		10	30.440	29.763	29.343	29.849	0.723	1.020	1.187	0.977
		15	28.730	28.097	26.920	27.916	1.323	1.400	1.890	1.538
		Mean	27.477	26.800	26.089	26.789	1.679	1.828	2.112	1.873
		Mean	24.461	23.796	23.141		2.580	2.687	2.974	
		Source	SEm±	CD at 1%			SEm±	CD at 1%		
		Nutrients (A)	0.0032	0.01188			0.0024	0.00891		
		Cultures (B)	0.0027	0.010025			0.0021	0.00779		
		Concentration C	0.0032	0.01188			0.0024	0.00891		
		A X B X C	0.0110	0.04084			0.0085	0.01819		

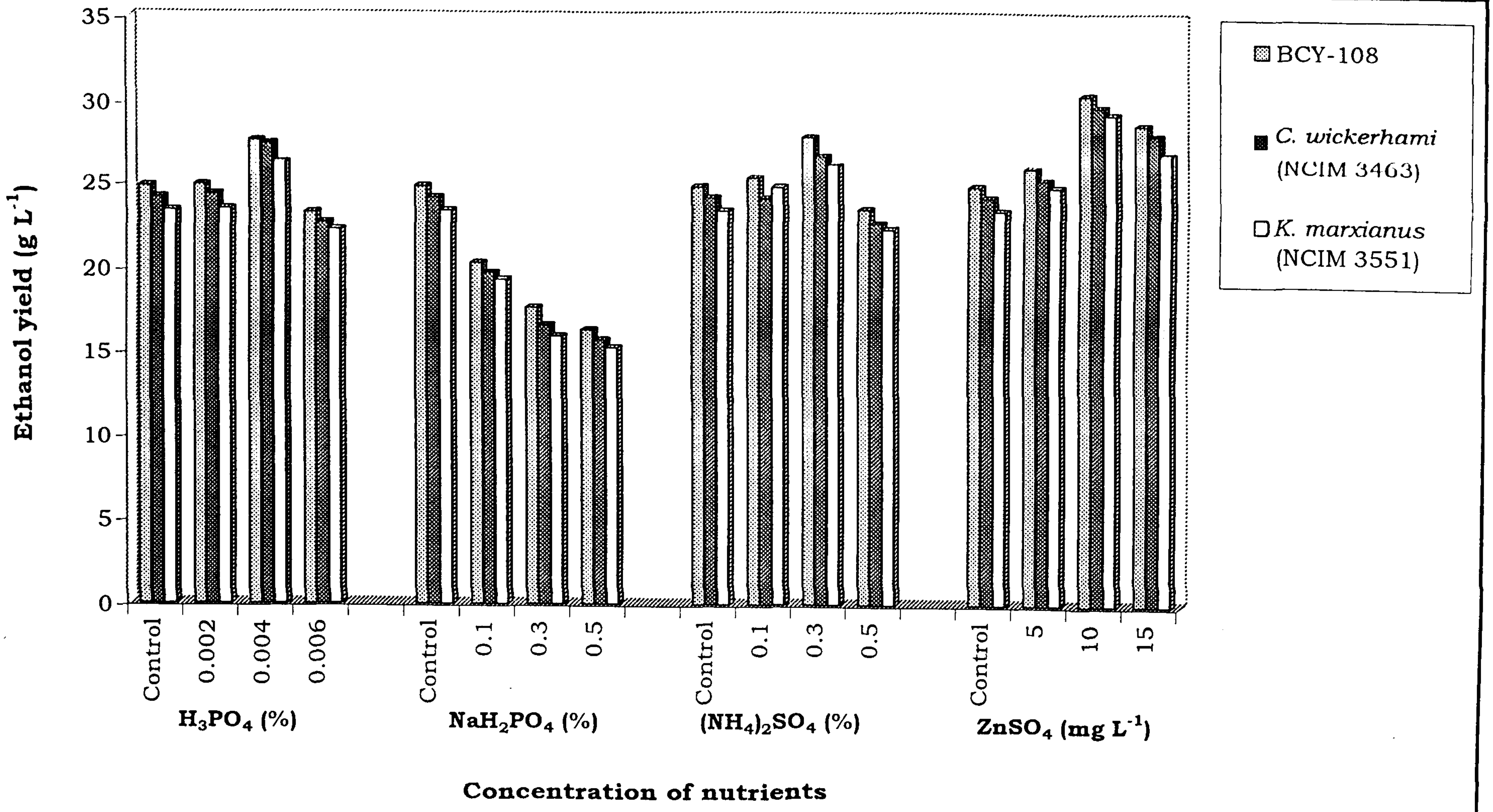


Fig. 8: Effect of nutrient supplementation on ethanol yield in the hydrolysate of deseeded pods of *S. saman*

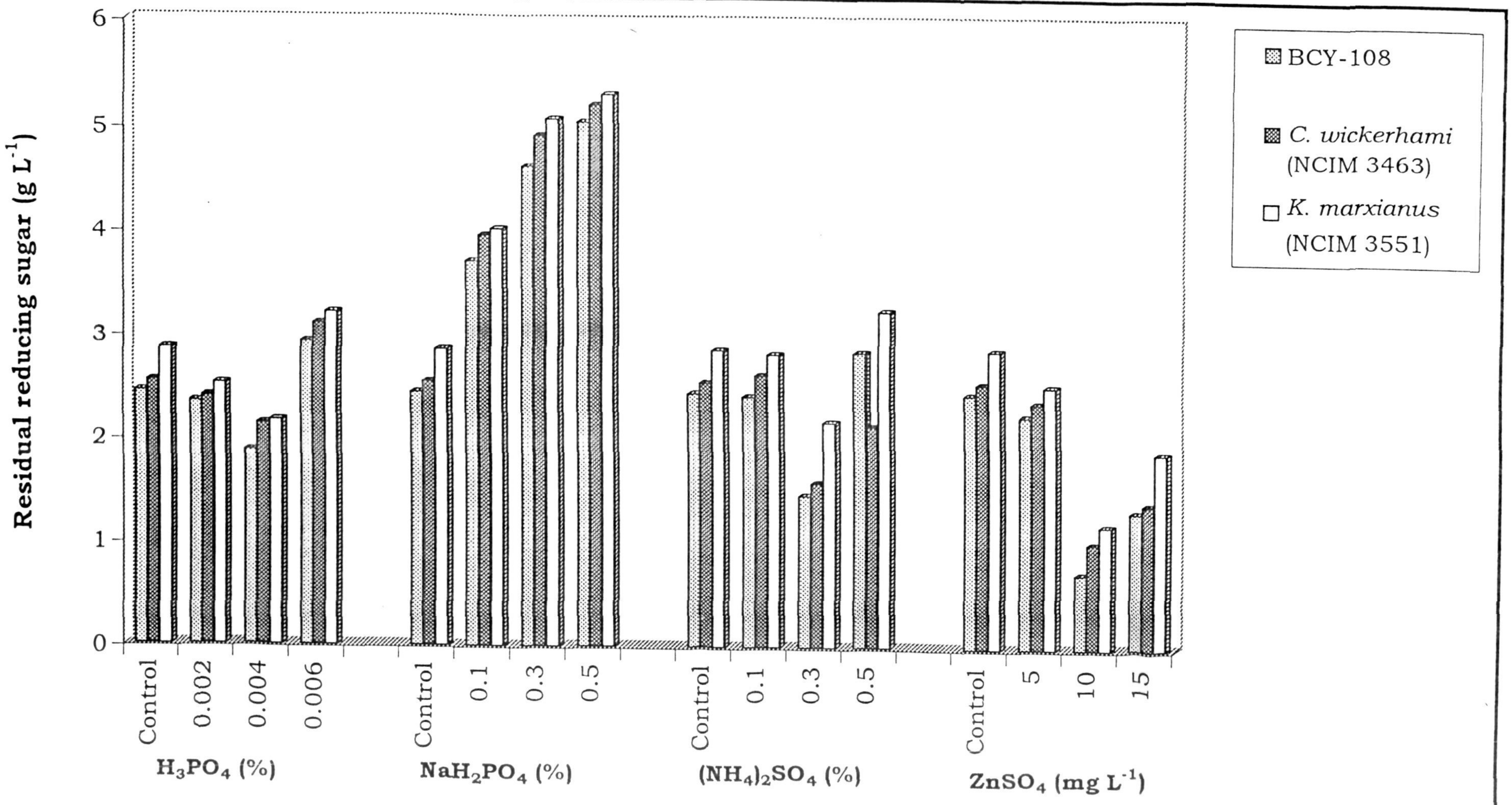


Fig. 9: Effect of nutrient supplementation on residual reducing sugar after fermentation of hydrolysate of deseeded pods of *S. saman*

significantly at 10 mg L^{-1} which was 0.977 g L^{-1}. As compared to control (unsupplemented), NaH_2PO_4 showed inhibition. Other nutrients, $ZnSO_4$, H_3PO_4 and $(NH_4)_2SO_4$ showed stimulation (optimum levels) in decreasing order for ethanol production as compared to control (24.092 g L^{-1}).

Among nutrients, the acid hydrolysate supplemented with $ZnSO_4$ had significantly higher ethanol yield which (26.789 g L^{-1}) found to be significantly superior as compared to other nutrients supplementation. Among nutrients, $ZnSO_4$ supplemented hydrolysate showed decreased residual sugar significantly which was 1.873 g L^{-1} (Table 13b). The significant effect of nutrients on ethanol and residual reducing sugars can be observed from Fig. 8 and 9.

4.14 PILOT SCALE PRODUCTION OF ETHANOL FROM PODS OF *S. saman*

A pilot scale fermentor of 3 L capacity was setup in three replications to know the performance of BCY-108 strain when scaled-up. The performance was evaluated in terms of ethanol yield, residual reducing sugar content and kinetics of yeast cell count. The efficient yeast strain, BCY-108 produced ethanol yield of 46.89 g/1.5 L (Table 14), while the residual sugar content in the acid hydrolysate was found to be 1.456 g/1.5 L. Accordingly, the yeast cell counts increased from 31.00 x 10^6 to 106.00 x 10^6 per ml of hydrolysate during aerobic growth of 24 h. Further yeast cell counts decreased at the end of fermentation period of 5 days (29.00 x 10^6).

Table 14 : Performance of pilot scale production of ethanol from acid hydrolysate of pods of *S. saman* in 3L fermentors

Sl.No.	Parameters	Values
1.	Ethanol yield (g/ 1.5 L)	46.89 (30.44 g L^{-1})
2.	Residual sugar (g/ 1.5 L)	1.456 (0.723 g L^{-1})
3.	Yeast cell count (x10^6 ml^{-1})	
	Initial	31
	After 24 hours	106
	End of fermentation (5 days)	29

DISCUSSION

V. DISCUSSION

Fossil fuels have two main insurmountable draw backs which limit their production and usage. One is that they are fast approaching depletion and the other is the environmental problems associated with their continued and increasing use. In the past few years, the price of the petroleum products and the natural gas have been inflated. A long term practical solution to this problem, is to convert a major source of continuously renewable non-fossil carbon like organic biomass.

A renewable nondepleting raw material that can be converted into fuel would assume a perpetual energy supply. As time passes and the fossil fuel shortage intensifies, renewable energy sources could eventually occupy a position of dominance. Ethanol is one of the biofuel which can substitute diesel upto 20 per cent. Presently, it has been implemented in a few states in India, that 5 per cent of ethanol is to be used along with diesel for transportation indicating the demand for ethanol. Ethanol can be produced from various biomass such as sudan grass, sweet sorghum, sugarcane, sugar beets, corn, wheat, barley, potato etc., which are available abundantly. In India, ethanol is produced by fermentation of molasses using strains of *Saccharomyces cerevisiae.* In recent years, due to the increase in cost of molasses, it has been emphasized to search alternate substrates for alcohol production. Some of the alternate biomass sources tried successfully are the pineapple, cannery waste (Nigam, 1999); starch (Verma *et al.*, 2000) and carob pods (Marakis and Marakis, 1996). From the preliminary studies, it was observed that pods of *S. saman* is rich in reducing sugars (Geeta *et al.*, 2001) indicating its possible exploitation for ethanol production. *S. saman* (Jacq.) Merr. is a tropical tree which is being listed as an economical plant. It is also well established that pods are used as an alternate feed stock for the cattle livestock (Ranjan, 1977). The inflorescence and pod bearing tree and the pods can be observed from Plate 1.

The tree is commonly planted as an ornamental shade plant along the road side. The present investigation was undertaken to optimize various pretreatment methods for maximum saccharification, screening of suitable efficient yeast cultures, optimization of fermentation parameters, and finally to study the pilot scale production of ethanol from the hydrolysate of deseeded pods of *S. saman*. Initially, the composition of the fresh deseeded pods were estimated. It was found to contain 40.6 per cent organic carbon, 1.05 per cent nitrogen, 6.56 per cent protein, 2.95 per cent tannin, 3.875 mg g^{-1} phenols, 20 per cent moisture, 80 per cent total solids, 460 mg g^{-1} total sugar, 210 mg g^{-1} reducing sugar and the C:N ratio was 38.66 and had a pH of 4.5 indicating its potentiality for fermentation. For the extraction of sugars, substrate:water ratio of 1:6 (w/v) was ideal for releasing the sugars into solution. One kg of deseeded pods when soaked with 6L of water for 10 h yielded 3.4 L of fermentable filtrate consisting of 19.77 g L^{-1} of reducing sugars. For obtaining further increase in sugars, suitable pretreatment methods were tried.

It was found that the reducing sugar content in the aqueous extract of deseeded pods of *S. saman* increased significantly with increase in incubation periods upto 8 hours, beyond which increase was nonsignificant. Marakis and Marakis (1996) obtained water soluble sugars in the aqueous carob extract consisting of sucrose (77%), fructose

The whole pods of *Samanea saman*

Deseeded pods of *Samanea saman*

Plate 1

(13.9%) and glucose (9%) with total water soluble sugar content of 54.7 per cent on dry weight basis. Similar water soluble sugars might have released in the present substrate also.

Since simple soaking in water for 8 hrs yielded only 1.977 per cent reducing sugars, other physical method like heat treatment was applied as the heat treatment is known to hydrolyse the substrates and release the sugars. The substrate was incubated with water (1:6 w/v) at various temperatures for 10 h to know the optimum temperature and soaking time. It was observed that the reducing sugar content increased significantly with increase in temperature upto 100°C. The highest was recorded at 100°C (23.559 g L^{-1}) followed by 80°C (23.504 g L^{-1}) as compared to 28±1°C (17.564 g L^{-1}) and 50°C (18.276 g L^{-1}), indicating the hastening process of hydrolysis at higher temperatures release of bound sugars (Van Walsum *et al.,* 1996). With respect to incubation periods, reducing sugar content increased significantly upto 8 hours, beyond which increase was non-significant, indicating that 8 h was optimum for releasing all the sugars. Relative to chemical pretreatment, hydrothermal pretreatment offers ecofriendly technology (Van Walsum *et al.*, 1996), producing much lower quantities of hydrolysate neutralization residues. Liquid hot water pretreatment, where biomass is exposed to pressurized hot water, appears to have the potential to recover most of the pentosans, and produce hydrolysate that results in little or no inhibition of glucose fermentation (Van Walsum *et al.*, 1996). Geeta *et al.* (2001) optimized the extraction of soluble reducing sugars from the deseeded pods of *S. saman* by hot water extraction and recorded maximum release of reducing sugars of 313 mg g^{-1}. When sugarcane bagasse was pretreated with hot water at temperature range of 170 to 230°C for 1 to 46 minutes, achieved

≥80 per cent xylan recovery with ≥80 per cent conversion by simultaneous saccharification and fermentation (Mark *et al.*, 2002). In the present study, maximum reducing sugars can be extracted at 80°C and 100°C with the optimum reaction time of minimum 8 h of incubation period.

It is well established that certain fungi are able to saccharify the substrate through their enzymes which cleave the complex polymer into simple sugars. Biological pretreatment is an attractive method as it could be cheap, efficient and ecofriendly. Hence, few fungi were inoculated to the solid substrate under both sterile and unsterile conditions. It was revealed that the reducing sugar content and per cent loss in weight was increased due to inoculation of different fungal cultures under both unsterilized and sterilized conditions. The highest reducing sugar content and per cent loss in weight of the substrate was recorded under unsterilized conditions, which accounts to 24.11 g L^{-1} and 50.13 per cent respectively, as compared to sterilized conditions. Probably, the destruction of sugars during sterilization or the development of natural selective microflora that could efficiently saccharify the substrate might have been responsible for releasing sugars. Among fungal cultures, *Aspergillus foetidus* (NCIM 515) was efficient in releasing the reducing sugar (24.26 g L^{-1}) and also loss in weight (48.32%), followed by *A. niger* (NCIM 616) which recorded 20.66 g L^{-1} and 44.00 per cent respectively. Zayed and Meyer (1996) examined *Trichoderma viride* and *Aspergillus niger* for their ability to produce fermentable sugars from cellulosic waste and achieved reducing sugar extraction of 27 g from 50 g delignified wheat straw at 25 to 30°C within 3 days. Similarly Geeta *et al.* (2002a) observed maximum saccharification of paddy straw due to inoculation of fungal cultures *viz., Phanaerochaete chrysosporium, Aspergillus foetidus, Aspergillus niger* and *Trichoderma viridae* for bioethanol production. Among fungal cultures *P. chrysosporium* was found to be superior in releasing fermentable sugars which yielded 534 mg L^{-1} and accounts to 53.4 per cent. Similarly per cent loss in weight of total solids was 24 per cent as compared to other fungal cultures. Increase in per cent loss in weight of the substrate can be ascribed to utilization of substrate as carbon source for growth of fungi and also due to metabolic activity of the fungus, resulting in weight loss of total solids. Similar studies on biological pretreatment for maximum saccharification of fermentable sugars was reported by Ramanathan (2000) in yam; Roche and Durand (1996) in sugar beet pulp; Scory and Bothast (1997) in cellulose, oat-spelt xylan, corn fiber and corn germ etc.

Chemical pretreatment of the pods was also compared to know the extent of hydrolysis for releasing the fermentable sugars. Alkali (NaOH) and acid (H_2SO_4) were used at various concentrations incubated upto 10 h. The reducing sugar content in the deseeded pods of *S. saman* was significantly influenced due to alkali pretreatment at different concentrations and incubation periods. The highest reducing sugar content was recorded at 1.50 per cent alkali concentration, which was 27.793 followed by 1.00 per cent (27.824 g L^{-1}). Further, it was found that it increased significantly upto 8h, beyond which increase in reducing sugar content did not differ significantly. Kahlon and Chaudhary (1988) reported that pretreatment of substrate with 1.0 per cent NaOH at 50°C with 2.5 per cent substrate concentration having pH 5.0 and incubation period of 4h, were optimum for maximum saccharification with cellulases. The chemical concentration and the exposure time are also dependent on the type of substrates. Sugarcane bagasse pith was pretreated with sodium hydroxide at concentrations 0.1 and 0.2 g g^{-1} pith at 50°C and 80 per cent moisture content and obtained maximum digestibility of 76 and 71 per cent respectively (Vazguez *et al.*, 1992). Azzam (1989) standardised various process conditions which included the contact time, the hydrogen peroxide concentration and the pretreatment temperature. Results showed that about 50 per cent of lignin and most of the hemicellulose content of cane bagasse was solubilized by 2 per cent alkaline hydrogen peroxide at 30°C within 8 h. Saccharification of this pulp residue with cellulase from *T. viridae* at 45°C for 24 h, yielded glucose with 95 per cent efficiency. Similar results were obtained by Vazguez and Diazeervantes (1994) in sugarcane bagasse pretreated with sodium hydroxide. Cao *et al.* (1996) reported that when corn cobs pretreated with 2.9 M NH_4OH, resulted in removing about 80 to 90 per cent of the lignin along with almost all the acetate from cellulosic residues and obtained high glucose yield of 92 per cent after enzymatic saccharification.

For comparison, sulphuric acid pretreatment was followed to know its efficiency in releasing the fermentable sugars. Dilute acid pretreatment, ammonia fibre explosion and lime pretreatment have emerged as particularly effective chemical methods (Himmel *et al.*, 1997; Dale *et al.*, 1996; Kaar and Holtzapple, 2000). Singh *et al.* (1984) studied the acid hydrolysis of bagasse and rice husk for the production of reducing sugars with sulphuric acid and optimized various process conditions like solid-liquid ratio, particle size, reaction time and concentration of acid. An acid concentration of 75 per cent (w/w) with soaking period of one hour, at temperature of 50°C, solid-liquid ratio of

1:12, particle size less than 417 microns for primary hydrolysis and heating time of 3 h at 100°C after dilution with 3 volumes of water, were found to be the optimum conditions, for the hydrolysis of bagasse and rice husk. Similarly, in the present study it was observed that the reducing sugar content in the acid hydrolysate of deseeded pods of *S. saman* was found to significantly improve at different acid concentrations and incubation periods. Reducing sugar content increased with increase in acid concentration upto 1.00 per cent (32.936 g L^{-1}) beyond which increase was not significant at 1.5 per cent (33.0678 g L^{-1}). Further, reducing sugar content increased with increase in incubation periods significantly upto 8 hours beyond which, it did not differ significantly. Chandrakant and Bisaria (2000) pretreated peanut shells (250 g L^{-1}) with one per cent H_2SO_4 at 103.5 k.pa. for 2 h resulting in the extraction of xylose (50 g L^{-1}), glucose (3.5 g L^{-1}), galactose (5 g L^{-1}), mannose (1 g L^{-1}) and arabinose (7 g L^{-1}). Ahring *et al.* (1999) recorded ethanol yield of 0.8±0.008 and 0.05±0.005 g L^{-1} from 0.67 and 2.2 per cent acid (H_2SO_4) pretreated hydrolysate of wheat straw and observed that highest acid concentration (2.2%) had an inhibitory effect on ethanol yield, whereas the low acid concentration (0.68%) increased the ethanol production. Recovery of sugars with 15 per cent sulphuric acid (Kahlon and Chaudhury, 1988), recovery of Xylan (Torget *et al.*, 1996) and xylose (Lee *et al.*, 2000) with 0.07 per cent dilute sulphuric acid and with 1.00 per cent sulphuric acid (Premidevi and Singh, 1995) from water hyacinth have been possible.

In the present study, pod substrate pretreated with 1.5 per cent acid exposed for 8 h was optimum, which might be due to the release of bound sugars from the available accessible sites as suggested by

Singh *et al.* (1984). From the results obtained, it could be concluded that all the methods of pretreatment had the potential to release the fermentable sugars when compared to fresh and simple aqueous extraction. But, dilute sulphuric acid pretreatment at 1.5 per cent was highly efficient in releasing the sugars. Further, neutralisation to pH 4.5 was also convenient by using potassium hydroxide pellets (approximately @ 2.2 g 100 ml^{-1}) which was not toxic when fermented with yeast cultures. Hence, substrate:acid extraction (1.5%) at 1:6 w/v was used in all further experiments. The yeast cultures were screened and evaluated for their potential to utilise the hydrolysate of pods of *S. saman* in terms of ethanol production and the residual sugar content after fermentation for 7 days. From the results, it was revealed that yeast cultures differed significantly in their ability to ferment acid hydrolysate of deseeded pods of *S. saman*. Among yeast cultures, BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 had recorded significantly higher ethanol yield of 20.257, 19.813 and 19.630 g L^{-1} respectively, and were significantly superior as compared to standard isolate CFTRI 101 (19.330 g L^{-1}), and other cultures. The uninoculated control did show 3.043 g L^{-1} of ethanol indicating the natural fermentation due to the competant air microflora. Residual sugar content recorded decreased significantly in the acid hydrolysate inoculated with BCY-108, *C. wickerhami* NCIM 3463 and *K. marximus* NCIM 3551 which were 3.561, 3.700 and 3.833 g L^{-1} respectively, as compared to other yeast isolates indicating their efficiency in utilising available sugars. Similar study conducted by Geeta *et al.* (2001) indicated maximum ethanol yield of 40.43 g L^{-1} by local isolate, BCY-107 followed by BCY-108 (36.06 g L^{-1});the other substrates like molasses (Panesar *et al.*,

2001) ; in sweet sorghum juice, (Jhawke *et al.,* 1983 and Waller and Hogaboam, 1983) were also highly potential. The difference in the performance of yeast strains may be due to the preferential utilization of pentose or hexose sugars present in the acid hydrolysate of deseeded pods of *S. saman.* The residual sugar content in the substrate also decreased significantly in the high ethanol producing strains which might be due to their high potential in utilization of reducing sugars.

A constant search for increasing ethanol yields has resulted in several approaches for improving the fermentation such as identifying efficient yeast strains, monitoring biochemical parameters, application of fed batch and continuous fermentations etc. Such parameters were also applied to optimise for the hydrolysate of pods of *S. saman.* The experimental set-up for fermentation was prepared as shown in Plate 2. In the present investigation, fermentation was one of the parameter tested. Among yeast cultures, BCY-108 recorded highest ethanol yield (16.568 g L^{-1}) as compared to *C. wickerhami* NCIM 3463 (16.304 g L^{-1}) and *K. marxianus* NCIM 3551 (16.034 g L^{-1}) throughout fermentation period. Similarly, residual reducing sugar content also decreased significantly in the acid hydrolysate inoculated with BCY-108 (4.822 g L^{-1}) indicating its efficiency in conversion of sugars. The ethanol yield further increased with increase in fermentation periods up to 9th day. However, it did not differ significantly between 7th and 9th day of fermentation (20.002 and 20.010 g L^{-1} respectively). Similar trend was recorded with respect to residual reducing sugar content between 7th and 9th day of fermentation (3.770 and 3.787 g L^{-1} respectively). Thus, the optimum period for utilization of the sugars was 7 to 9 days. The observation is in accordance with the findings of previous studies in other substrates.

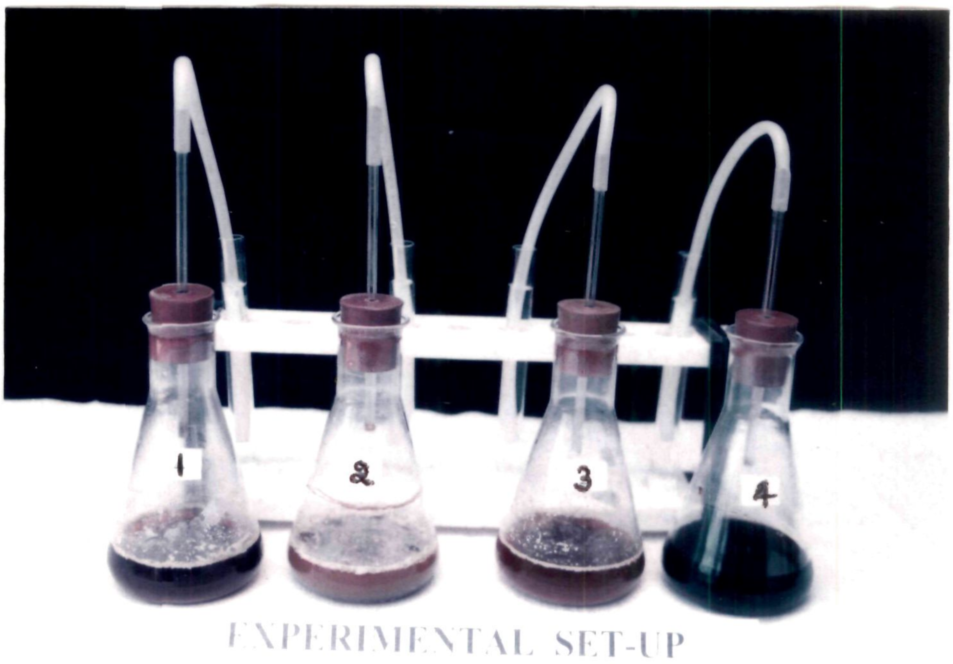


Plate 2 : Experimental set-up for standardising optimum parameters for ethanol production from *S. saman*

Zossi *et al.* (1990) achieved highest fermentation efficiency of 96.916±0.83 per cent, when fermented sugarcane juice for 7 days at pH 6.0. On the contrary, Suresh *et al.* (1999) obtained maximum ethanol yield of 2.9 per cent (v/v) after 5 days of fermentation from damaged sorghum and rice grains. Marakis and Marakis (1996) studied the effect of six different fermentation periods *viz.*, 0, 24, 48, 72, 90 and 100 hours on ethanol production from aqueous carob extract and achieved maximum alcohol concentration of 4.75 per cent (v/v) at 100 hours of fermentation period. Similarly, Sreenath and Jeffries (2000) recorded maximum ethanol concentration of 34.8±2.42 g L^{-1} during 3 to 5 days of fermentation period from wood hydrolysate at pH of 5.5 to 7.0 and temperature of 25 to 27°C respectively. Similar results were obtained on the fermentation period for maximum production of ethanol was reported in yam (Ramanathan, 2000), from starch medium (Verma *et al.*, 2000) and in algae (Shirai, 1998).

A constant ethanol yield between 7th and 9th day of fermentation can be attributed to the exhaustion of available sugars or less tolerance of yeast cells to ethanol concentration. Any increase in ethanol production might have been accumulated in the cells as suggested by Burrill *et al.* (1983). Such accumulation inhibits some intermediate activities (formation of RNA or proteins) that inturn result in delayed growth inhibition (Jobes *et al.*, 1986) and becomes more toxic to growth of the cells (Hagerdal *et al.*, 1994). Another parameter, pH of the substrate was tested at various levels to know the optimum level for ethanol production. The highest ethanol yield was recorded at pH 4.5 (19.953 g L^{-1}) beyond which it decreased significantly. Among yeast cultures, BCY-108 recorded highest ethanol yield of 9.656 g L^{-1}. A matching trend was recorded with respect to residual reducing sugar content which decreased significantly in the acid hydrolysate inoculated with BCY-108 at pH 4.5. Marakis and Marakis (1996) have obtained maximum ethanol concentration of 5.8 per cent (v/v) at pH 4.5 from aqueous carob extract after 120 hours of fermentation. Similarly, Nimbkar *et al.* (1989) observed maximum alcohol concentration of 12.45 per cent (v/v) at pH 4.5 from juice of sweet sorghum through fermentation by *Saccharomyces cerevisiae* strain 3319. Azzam *et al.* (1988) obtained maximum ethanol yield of 26.8 g L^{-1} with the fermentation efficiency of 82.3 per cent at pH 4.5 from molasses medium (22% sugars). Similar results were reported in sweet sorghum juice (Swaminathan, 1987); in cane molasses (Garcia and Suarez, 1992); in guava pulp (Suchi Srivastava *et al.*, 1997). The decrease in ethanol yield at higher pH could be attributed to either ethanol respiration or low levels of inhibition as a consequence of potassium acetate formation in the medium during buffering when potassium hydroxide was used to maintain the pH (Sreenath and Jeffries, 2000). With respect to temperature, ethanol yield was increased significantly upto 30°C (20.004 g L^{-1}) beyond which it decreased. Accordingly, decrease in residual reducing sugar content (3.952 g L^{-1}) was observed. Verma *et al.* (2000) observed that maximum ethanol concentration of 21.8 g L^{-1} at optimum temperature of 30°C in 48 hours. Similarly, Panesar *et al.* (2001) optimized the fermentation temperature as 30°C for ethanol production from molasses and also Nimbkar *et al.* (1989) obtained maximum alcohol concentration of 12.45 per cent (v/v) at 30°C out of 25, 30 and 35°C from sweet sorghum juice by *Saccharomyces cerevisiae* 3320. When cane juice was fermented with *Z. mobilis*, the decrease in ethanol yield at higher temperature indicated an increased production of aldehydes, lactic acid and an increased sensitivity of the strain to ethanol inhibition (Singh and Jain, 1994). Further, high temperature is thought to cause increased fluidity in membranes gradually. Both heat and ethanol cause membrane disordering and protein denaturation (Piper, 1995), in addition to inhibiting glycolysis and enhancing mutations (Van Uden, 1984) that alter plasma membrane ATPase activity, which is responsible for maintaining the proton gradient across the plasma membrane (Serrano, 1991). Both stresses also increase the permeability of the plasma membrane, resulting in an increased passive proton influx that acts to dissipate the electrochemical potential gradient that the cell maintains across this membrane, which inturn adversely affects nutrient uptake, maintenance of the K balance and the regulation of intracellular pH (Serrano, 1991). Similar mechanism might have occurred in the present study resulting in decreased ethanol yield at higher temperatures.

The optimization of inoculum size was essential to know its effect on fermentation time and ethanol production. Roberto *et al.* (1995) used higher inoculum density to minimise inhibitory effects of the acetic acid present in the hydrolysate of agro residues. Hence, various inoculum levels were tried. Ethanol yield increased significantly with increase in inoculum size up to 1.5 per cent (24.131 g L^{-1}), beyond which increase did not differed significantly. Similar trend was noticed with respect to decrease in residual sugar content (2.573 g L^{-1}) at 1.5 per cent inoculum. The carbon dioxide production seized at 5 days of fermentation in 1.5 per cent inoculum as compared to 1.0 per cent inoculum which showed 7 days in completing fermentation. Hence an optimum period of

5 days fermentation at 1.5 per cent inoculum was followed. Kaur and Kocher (2002) optimized inoculum size and temperature of 10^8 cells ml^{-1} and 30°C respectively, for ethanol production from molasses by *S. cerevisiae* MK1 and obtained maximum ethanol yield of 89.8 g L^{-1} with 88 per cent fermentation efficiency. Nimbkar *et al.* (1989) used inoculum volumes of 0.5, 1.0 and 2.0 ml 100 ml^{-1} of unsterilized sweet sorghum juice for *S. cerevisiae* and achieved optimum ethanol concentration of 12.23 per cent (v/v) at 2.0 ml. Similarly, Mathur *et al.* (1986) obtained alcohol concentration of 2.18 per cent (w/v) from whey inoculated with 1.00 per cent of *S. cerevisiae* after 18 hours of fermentation period at pH and temperature of 4.0 and 22°C respectively. On the contrary, Ramanathan (2000) could achieve maximum alcohol recovery of 5.85 per cent at 5 per cent yeast concentration during fermentation of yam to ethanol. Similar observations were found by Gibbons and Westby (1986) in fodder beets. Kahlon and Chaudhary (1988) optimized 8 per cent yeast inoculum for fermentation of water hyacinth for 36 hours. Similar studies on the effect of different inoculum levels for maximum production of ethanol was reported in sucrose medium (Singh and Jain, 1994); in sweet sorghum juice (Swaminathan *et al.*, 1987); in Molasses (Mendoza and Raymundo, 1990). The increase in ethanol yield at 1.5 or 2.00 per cent inoculum size can be attributed to the increase in cell density responsible for oxidative metabolism there by maximum conversion of sugars is possible. Due to the limitation in oxygen, fermentative metabolism would then set earlier as compared to that of lower inoculum size.

The addition of nutrients or stimulants could be an alternate approach for enhancing the ethanol production as they will provide ideal

C:N ratio or optimum nutrients or to activate the enzymes. Hence, ammonium sulphate was used to supplement nitrogen and orthophosphoric acid and di-sodium hydrogen phosphate as phosphorus source were used at various concentrations. Zinc sulphate was also used as a stimulant for enzyme activation. Among the nutrients tested, ethanol yield was positively influenced when acid hydrolysate was supplemented with zinc sulphate ($ZnSO_4$) at different concentrations. Maximum ethanol yield was noticed when $ZnSO_4$ supplemented @ 10 mg L^{-1} (26.789 g L^{-1}) and inhibited at higher concentration. The residual sugar content also decreased significantly (1.873 g L^{-1}) due to the efficient utilization of sugar. Sreenath and Jeffries (2000), obtained maximum ethanol yield of 26-30 g L^{-1} by supplementation of wood hydrolysate medium with $ZnSO_4$ @ 10 mg L^{-1}. Similarly, Geeta *et al.* (2001) studied the effect of $ZnSO_4$ at various concentrations *viz.*, 0, 10 and 15 mg L^{-1} on ethanol production from the pods of *S. saman* and achieved maximum ethanol concentration at 10 mg L^{-1} $ZnSO_4$. The increase in ethanol production caused by addition of Zn was due to high activity of zinc-dependent Alcohol dehydrogenase (ADH) in the fermentation pathway. ADH have been extensively studied in *S. cerevisiae* where it occurs as four isozymes and has been found to be Zn dependent (Hagerdal *et al.*, 1994). Addition of Zn @ 10 mg L^{-1} to acid hydrolysate did increase rates of sugar utilization and ethanol production and also observed that zinc acts as a stimulant for enzyme and inturn alcohol yield.

When nitrogen was supplemented as ammonium sulphate using molasses medium at concentrations of 0.1 per cent using *Z. mobilis* strain B-61147, ethanol concentration at hourly intervals was 65-70 g L^{-1}, 54 g L^{-1} and 75.1 g L^{-1}, 68.8 g L^{-1} (Iida *et al.*, 1993). Similarly, Anantha and Gunasekaran (2000) obtained higher ethanol yield of 44.2 and 54.9 g L^{-1} by supplementation of liquefied cassava starch with ammonium sulphate and yeast extract at concentrations of 1 and 10 g L^{-1} respectively, as compared to that of unsupplementation (36.5 g L^{-1}). In the present study, $(NH_4)_2$ SO_4 at concentration of 0.3 per cent yielded 26.966 g L^{-1} alcohol. The higher concentration inhibited alcohol production. Similar observations were made in cane molasses (Huertas, 1990); and in sweet sorghum juice (Swaminathan *et al.*, 1987). Marakis and Marakis (1996) supplemented aqueous carob extract with both sodium dihydrogen phosphate and ammonium sulphate at the rate of 0.1 and 0.3 per cent respectively and fermented with *S. cerevisiae*. A maximum ethanol concentration of 5.8 per cent (v/v) after 120 hours of fermentation was obtained. Israilides *et al.* (1998) obtained higher ethanol yield of 30.8 g L^{-1} when the grape skin pulp extract was supplemented with dipotassium hydrogen orthophosphate (K_2HPO_4) and ammonium nitrate (NH_4NO_3) at the rate of 0.1 per cent each respectively, compared to ethanol yield without supplementation (23.3 g L^{-1}). On the contrary, Cuesta and Cornejo (1988) proved that, addition of equal amounts of H_3PO_4 and $(NH_4)_2SO_4$ upto 0.7 per cent each increased fermentation rate and level of ethanol production during the whey fermentation by *Candida psuedotropicalis* IFI-1268. Similarly, Ramanathan (2000) proved that each tonne of cassava requires the use of 200 g each of tripple super phosphate and ammonium sulphate and 20 g magnesium sulphate also for fermentation of cassava to ethanol (25-30%).

All the optimized parameters we applied simultaneously in the lab-scale pilot fermentor of 3 L capacity. The acid hydrolysate of 1.5 L with a pH of 4.5 was maintained in a 3 L bottle fermentor (Plate 3). Zinc sulphate was supplemented @ 10 mg L^{-1} and inoculated with 1.5 per cent BCY-108 yeast strain. At the end of incubation period of 5 days, performance was evaluated in terms of ethanol and residual reducing sugars. The observations recorded clearly revealed that, BCY-108, an efficient yeast culture produced highest ethanol yield of 46.89 g/1.5 L. While the residual sugar content in the acid hydrolysate was 1.456 g/1.5 L. The yeast cell counts increased from 31.00 to 106.00 x 10^6 per ml of hydrolysate during aerobic growth. Further, yeast cell counts decreased at 5th day of fermentation (29.00 x 10^6). Hermann *et al.* (1986) fed the deproteinized whey with lactose content of 23 per cent continuously to 3L fermentor bottle at a flow rate of 250 ml h^{-1} with *Z. mobilis* immobilized with sodium alginate and could obtain ethanol yield of 41 to 42 g L^{-1}. Similarly, Mohammad *et al.* (1999) examined batch and fed batch fermentations which were tested in a lab scale (3.3 L) anaerobic bioreactor and found fed-batch technique with a suitably adjusted feed rate, was possible to completely ferment the glucose and mannose sugars. On the other hand, Taylor *et al.* (2000) demonstrated ethanol production on a pilot scale for the conversion of high solids saccharified corn mash to ethanol by continuous fermentation and carbon dioxide trapping. Similar observations were made by Saucedo and Karim (1996) for xylose medium; Perlot *et al.* (1989) for water hyacinth hemicellulose and xylose rich sugar mixtures; Bark *et al.* (1992) for sugar beet molasses.

Thus, the acid hydrolysate of pods of *S. saman* could be an ideal substrate for ethanol production when the various parameters are maintained at optimum levels. The local yeast isolate, BCY-108 strain

Plate 3 : Pilot-scale lab fermentor

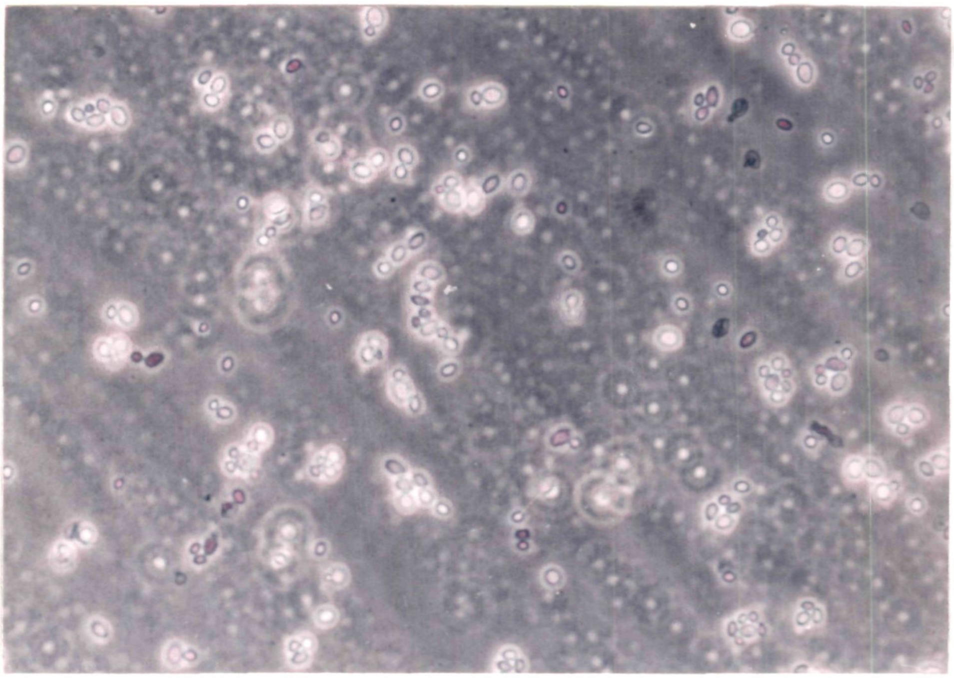


Plate 4 : Efficient yeast strain BCY-108 x 40X

has proved to be superior (Plate 4) in utilization of the substrate. The complete processing of the pods for ethanol production is being presented in the Fig. 10. Further it is essential to know the recovery efficiency of absolute ethanol yield from such substrate.

Future line of research

1. Alternate processing methods which do not involve chemicals could be tried to make the technology more ecofriendly and profitable.
2. A combination of various other nutrients might further enhance the ethanol production.
3. Designing of pilot scale fermentors and distillation unit for obtaining absolute ethanol.
4. Characterization and identification of the yeast BCY-108 strain
5. Genetically engineered microorganisms could be tried for utilization of the non reducing sugars for complete conversion of the sugars.

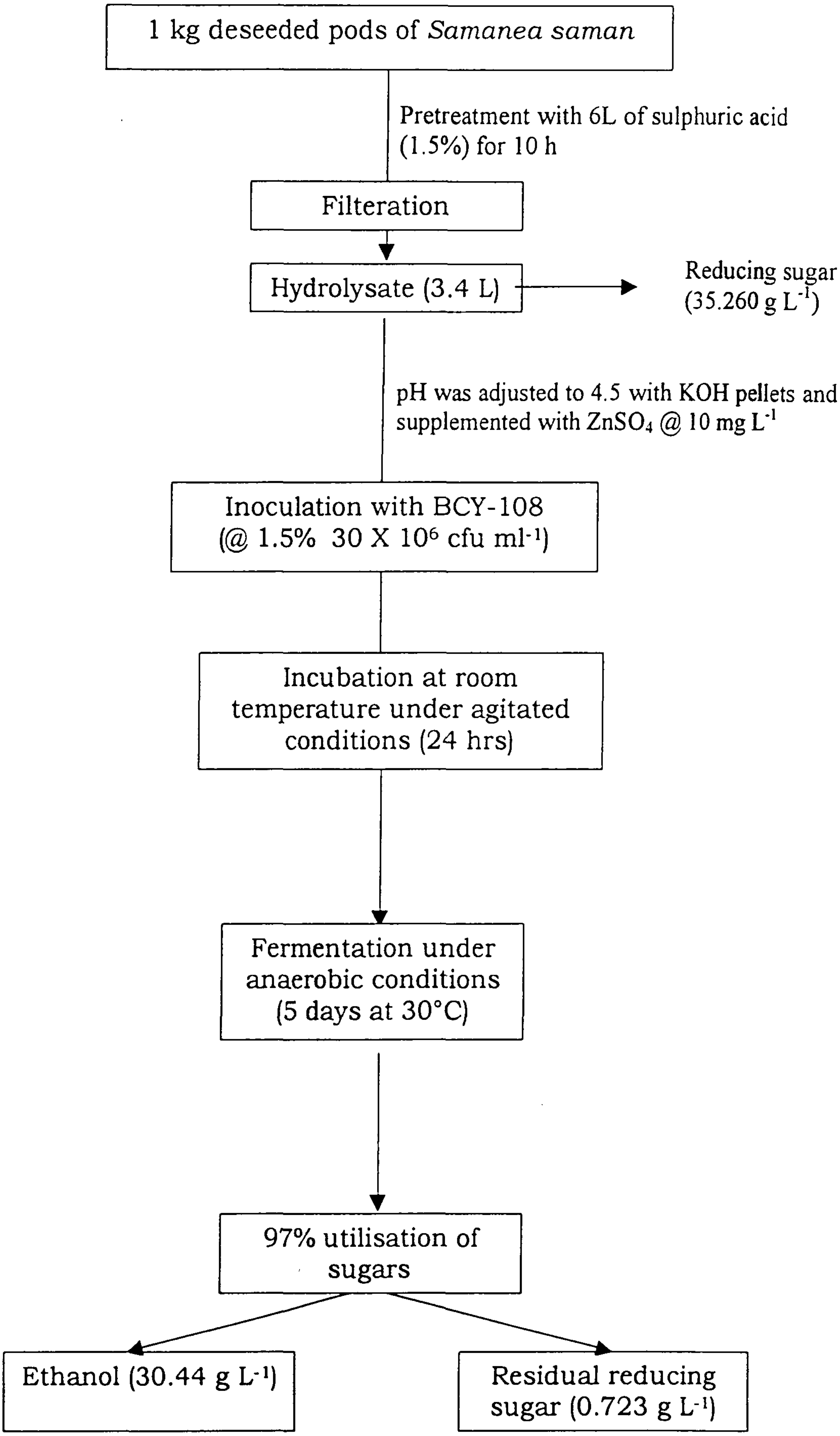


Fig. 10 : Flow diagram depicting the processing of the pods of *S. saman*

SUMMARY

VI. SUMMARY

The concept of blending ethanol with petrol or diesel @ 20 per cent has been practically introduced recently to conserve the natural resources and reduce huge amount of foreign exchange incurred for importing petrol and diesel. At present, majority of our distilleries are using molasses, a byproduct of sugar industries. Due to the decrease in availability and high cost, alternate sources need to be identified. The agricultural crops such as sweet sorghum, beet, corn, potato have been promising alternates. But, their lengthy processing techniques increase the cost of production. The pods of *S. saman,* a tropical tree could be best alternate for producing ethanol as it contains the required nutrients in appreciable quantities. Therefore, the present investigation was carried out with the aim of optimising the conditions for obtaining maximum reducing sugars and ethanol.

Samanea saman (Jacq.) Merr. is a tropical tree listed as an economic plant. It is generally planted as an ornamental and shade tree all along the road sides. The University Campus has such trees widely spread all over. The initial constituents of fresh pods indicated mainly 40.6 per cent of carbon, 1.05 per cent of nitrogen, 460 mg g^{-1} of total sugars and 210 mg g^{-1} of reducing sugars. For further release of bound reducing sugars, pretreatment methods were tried, which include cold and hot aqueous extraction, alkali, acid and fungal pretreatment. All the pretreatment methods did influence in releasing the reducing sugars within 8 h of respective treatments. Aqueous extraction at 100°C, released maximum reducing sugars which was not significant to 80°C as compared to cold extraction. In biological pretreatment, *A. foetidus*

(NCIM 515) recorded significant release of reducing sugars and increase in per cent loss of weight followed by *A. niger* (NCIM 616) irrespective of the conditions. However, sterile conditions showed inhibition in releasing sugars as compared to unsterile conditions which can be attributed to the destruction of sugars at high temperature during sterilisation.

The chemical pretreatment (sodium hydroxide and sulphuric acid) was also adopted to know its effect on releasing sugars. As the concentration of alkali or acid increased, there was increase in sugars (27.824 and 33.067 g L^{-1} respectively) with increase in time of exposure. The optimum period was 8 h. Further increase in sugars was not significant. Hence, 1.5 per cent acid extraction was followed in further experiments as other methods were not effective and were not accessible for neutralising the pH for subsequent process of fermentation.

The local yeast isolates (7) along with standard yeast cultures (13) were screened to know their potential in utilising the substrate. The yeast cultures differed significantly in their ability to ferment ethanol yield which ranges from 12.207 to 20.257 g L^{-1}. Among yeast cultures, BCY-108 *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551 were found to be efficient which recorded 20.257, 19.813 and 19.630 g L^{-1} respectively. These were significantly superior as compared to standard isolate, *S. cerevisiae* CFTRI 101 (19.330 g L^{-1}) and other yeast cultures and uninoculated control (3.043 g L^{-1}). The residual sugar content after fermentation also decreased significantly with inoculation of BCY-108, *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM-3551 which recorded 3.567, 3.700 and 3.833 g L^{-1} respectively, as compared to other treatments and uninoculated control (8.767 g L^{-1}). Hence, these three cultures were used for further optimisation of parameters. Ethanol yield increased significantly due to different fermentation periods upto 7 days beyond which it did not differ significantly between 7th and 9th day (20.002 and 20.010 g L^{-1}). Accordingly, the residual sugar content was also reduced. But, it was non significant among the cultures.

The pH had significant influence on ethanol yield in the acid hydrolysate of deseeded pods of *S. saman*. The highest ethanol yield was recorded at pH 4.5 (19.953 g L^{-1}) beyond which it decreased significantly at pH 6.5. Among yeast cultures, BCY-108 proved its efficiency in producing ethanol yield of 9.656 g L^{-1} and was significantly superior to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551. The residual sugar content also was affected due to different pH levels, which ranges from 5.907 to 3.869 g L^{-1} at pH 3.5 and 4.5 respectively.

Ethanol yield had positive influence due to the variation in temperature and inoculation with efficient yeast cultures. The ethanol yield increased significantly upto 30°C beyond which it decreased significantly. The maximum ethanol yield of 20.04 g L^{-1} was recorded at 30°C. Among yeast cultures, BCY-108 recorded highest ethanol yield of 16.856 g L^{-1} as compared to *C. wickerhami* NCIM 3463 (15.888 g L^{-1}) and *K. marxianus* NCIM 3551 (15.311 g L^{-1}). While the residual sugar content also decreased significantly at 30°C with inoculation of BCY-108 (3.952 g L^{-1}) and *K. marxianus* NCIM 3551 (22.163 g L^{-1}).

The inoculum size had significant influence on ethanol yield which increased with increase in inoculum level upto 1.5 per cent. Further increase in ethanol yield did not change significantly, but highest was recorded at 1.5 and 2 per cent inoculum level which accounts to 24.131

and 24.154 g L^{-1} respectively. While residual sugar content also decreased significantly at 1.5 and 2 per cent inoculum level, it did not differ significantly (2.573 and 2.549 g L^{-1} respectively). Among yeast cultures, BCY-108 recorded highest ethanol yield (23.407 g L^{-1}) as compared to *C. wickerhami* NCIM-3463 (22.77 g L^{-1}).

The acid hydrolysate of deseeded pods of *S. saman* inoculated with efficient yeast cultures along with supplementation of nutrients had positive influence on ethanol yield and reducing sugar content. Among yeast cultures, BCY-108 was found to be efficient in producing ethanol yield as compared to *C. wickerhami* NCIM 3463 and *K. marxianus* NCIM 3551. Among nutrients, when acid hydrolysate was supplemented with zinc sulphate @ 10 mg L^{-1} had significantly higher ethanol yield (29.849 g L^{-1}) followed by orthophosphoric acid @ 0.004 per cent (27.228 g L^{-1}), ammonium sulphate @ 0.3 per cent (26.966 g L^{-1}) and sodium dihydrogen orthophosphate @ 0.1 per cent (19.809 g L^{-1}). Accordingly, the residual sugar content also decreased significantly similar to the response of ethanol production for nutrients.

Based on the optimum parameters obtained and the performance of yeast strain BCY-108, a pilot scale experiment was setup using 3L fermentor designed in the lab. The optimum conditions of pH 4.5, fermentation time of 5 days, temperature of 30°C, inoculum size of 1.5 per cent and supplementation of zinc sulphate @ 10 mg L^{-1} were maintained. It was evaluated in terms of ethanol yield and residual sugars. Maximum ethanol yield (46.89 g/1.5 L) was recorded with residual reducing sugar content (1.456 g/1.5 L). The yeast cell counts increased due to the aerobic growth at the beginning (106.00 x 10^6 ml^{-1})

and later it decreased on 5th day at the end of fermentation, which can be attributed to the exhaustion of utilisable sugars and ethanol toxicity.

Thus, deseeded pods of *S. saman* can be easily processed for releasing maximum reducing sugars which can be almost completely fermented (97.23%) to ethanol with the efficient yeast strain of BCY-108, at the derived conditions.

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VII. REFERENCES

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APPENDIX

APPENDIX I

1. Potato Dextrose Agar media (PDA)

Potato - 200 g
Dextrose - 20 g
Yeast extract - 0.10 g
Agar - 20 g
Distilled water - 1000 ml.
pH - 6.0

Preparation : 200 g of peeled potatoes were cut into small pieces and suspended in 1000 ml of distilled water and steamed for 30 min. The extract was obtained by filtering through the muslin cloth and final volume made upto 1000 ml.

2. Malt extract – Glucose - Yeast Extract - Peptone (MGYP) agar medium

Yeast extract - 3.0 g
Malt extract - 3.0 g
Peptone - 5.0 g
Glucose - 10.0 g
Agar - 16.0 g
Distilled water - 1000 ml
pH - 6.4-6.8.

BIOETHANOL PRODUCTION FROM THE PODS OF *Samanea saman* (Jacq.) Merr.

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Abstract

Ethanol is being blended with petrol or diesel @ 20% in transportation in order to conserve the natural resource and reduce the cost. Molasses is the only major source for producing ethanol, which is available at a high cost. From the preliminary observations, it was evident that the pods of *S. saman* consist of reducing sugars in appreciable amount. Hence, the present study was conducted to know its potential and to optimise the parameters for ethanol production. Various pretreatment methods were tested for obtaining maximum reducing sugars. The aqueous extraction with and without heat treatment, fungal and alkali pretreatment methods yielded less amount of reducing sugars as compared to acid pretreatment. Acid pretreatment @ 1.5% was found to be effective in releasing reducing sugars. Increase in reducing sugar was significant up to 8 hrs of incubation period, beyond which increase was not significant. Hence, acid pretreatment was followed for further studies for ethanol production.

Among the yeast strains screened, BCY-108, *Candida wickerhami* NCIM 3463 and *Kluveromyces marxianus* NCIM 3551 recorded highest ethanol yield with corresponding decrease in residual reducing sugar. These three strains were further used to optimise fermentation parameters. The fermentation period of 5 days, pH of 4.5, temperature of 30°C and 1.5 per cent inoculum level were optimum for production of maximum ethanol as compared to control. The BCY-108 yeast strain was efficient as compared to other yeast cultures. For further enhancing the ethanol production, acid hydrolysate was supplemented with zinc sulphate, ammonium sulphate, orthophosphoric acid and sodium dihydrogen orthophosphate. Among these nutrient supplementation, $ZnSO_4$ @ 10 mg L^{-1} stimulated maximum ethanol yield of 29.849 g L^{-1}. Based on these parameters, a pilot scale fermentor of 3L capacity was set in three replications. The hydrolysate containing 35.26 g L^{-1} of reducing sugars yielded an average of 30.44 g L^{-1} of ethanol and 0.72 g L^{-1} of residual reducing sugars, indicating 97 per cent conversion. Thus, it can be concluded that pods of *S. saman* can be successfully used for ethanol production.