ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC YEASTS FOR BIOETHANOL PRODUCTION

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SARAT CHANDRA SAHOO

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
COLLEGE OF AGRICULTURE, DHARWAD
UNIVERSITY OF AGRICULTURAL SCIENCES,
DHARWAD – 580 005

JULY, 2013

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INTRODUCTION

Energy is an important factor for the economic development of a nation. One of the greatest challenges for society in the 21st century is to meet the growing demand of energy for transportation, heating and industrial process and to provide raw material for the industry in a sustainable way. Bio fuel is globally considered as the sustainable and eco-friendly source of energy to enhance national energy security and to decrease dependence on imported fossil fuel.

India and China are expected to account for 17 per cent of global liquid fuel consumption surpassing total consumption of entire Europe by 2025 (Gehlhar *et al*., 2010). The present rate of petrol consumption results in depletion of natural reserves. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050. India consumes more than 250 million tonnes of fossil fuels every year. This comprises of approximately 40 million tonnes of diesel. Thirty five countries in the world are using 10 per cent alcohol with petrol and 15 per cent alcohol with diesel. All above 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).

India is ranked fifth in the world after China, Japan, Russia and the U.S. in terms of fossil fuel consumption (Shukla, 2005). 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. In India, there are 295 distilleries producing 1058 million litres of alcohol as against annual consumption of 1266 million litres (Arbatti, 2001). Govt. of India is planning to bring into effect 20% blending of petrol with bio-ethanol by 2017, it is important to anticipate ethanol demand, so that necessary measures may be undertaken to achieve targets.

Bio-ethanol is one such principal alternate fuel that can substitute petrol. Apart from this it has also much lower contribution to green house gases compared with fossil fuels. Ethanol or ethyl alcohol (C_2H_5OH) is a clear colourless liquid, it is biodegradable, low in toxicity and cause little environmental pollution. It is a high octane fuel has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline we can also oxygenate the fuel mixture so it burns more completely and reduces polluting emissions. The admixture of petrol- alcohol and diesel- alcohol are known as gasohol and diesohol respectively. Ethanol fuel blends are sold in the United States. The most common blend is 10% ethanol and 90% petrol (E10).

There is a copious amount of cellulosic biomass worldwide that can be exploited for ethanol production. The steps required for this process include pre-treatment of biomass, the use of cellulolytic enzymes for depolymerization of carbohydrate polymers into fermentable constituents, and the use of robust fermentative microorganisms for ethanol production (Soccol *et al*., 2010; Chandel and Singh, 2011). The cost of cellulosic ethanol is a major obstacle. However, integration of the saccharification of biomass and fermentation of released sugars into simultaneous saccharification and fermentation (SSF) has made a crucial impact on lowering ethanol production costs (Olofsson *et al*., 2008).

Therefore agriculture and forest residues as well as dedicated crops can be the sources for the production of ethanol. Cellulose is a major component of the cell wall of plants and the most abundant and the renewable carbohydrate. Hence practical and cost effective processes for bio-ethanol production from cellulose are highly desired.

Few yeast species are capable of utilizing many different substrates including monosaccharide, disaccharides, trisaccharides, ethanol and organic acids. *S. cerevisiae* is mainly used for bio-ethanol production because of its high ethanol productivity, high ethanol tolerance, high endurance in industrial process and simplicity of genetic engineering. At present ethanol production from agro residues is by two step process i.e. saccharification and fermentation which makes it more costly and time consuming process.

It is essential to look for yeast strains that have the ability to utilise cellulose and ethanol production, so that alcohol can be obtained from a single step. Hence, the present investigation was undertaken with following objectives.

1. Isolation and screening of yeast species for hydrolysis of cellulose.
2. To study the performance of these efficient yeast isolates in bioethanol production from crop residues.
3. Quantification of cellulose hydrolysis by the yeast isolates.

REVIEW OF LITERATURE

The most common renewable fuel produced today is ethanol derived from corn grain (starch) and sugarcane (sucrose) and cellulose and the annual worldwide production of cellulose alone accounting to 1011 metric tonnes makes it a good substrate for bioethanol. Presently molasses is being used in many distilleries for its ideal sugar content suitable for production of ethanol. But, its availability at high cost could increase the cost of production .It is expected that there will be limits to the supply of these raw materials in the near future; therefore, cellulosic biomass is considered as an attractive feed stock for future supplies of ethanol (Wyman *et al.,* 2005).

Cellulosic biomass is one of the virtually inexhaustible and easily available renewable sources of energy. The abundantly available lignocellulosics require pre-treatment for obtaining fermentable sugars and conversion of the same to ethanol. The various approaches include pre-treatment of substrates, suitable bacteria, fungi and yeast strains for hydrolysis, yeast strains for fermentation and purification of ethanol. One of the important approach is the production of ethanol in a single step which save time as compared to two step process. The literature pertaining to these aspects are being reviewed here.

2.1 Bioethanol

Ethanol or ethyl alcohol (C_2H_5OH) is an important organic chemical because of its unique properties and therefore can be used widely for various purposes. Under normal conditions, ethanol is a volatile, flammable, clear, colourless liquid, miscible in both water and non-polar solvents (Lang *et al.,* 2001). As reported by Lynd and Zhang (2002), *S. cerevisiae* is the most popular microorganism used for ethanol production due to its high ethanol yield and high tolerance to high ethanol concentration. The ethanol produced is mostly used as fuels (92%), industrial solvents and chemicals (4%) and as beverages (4%).

Howard *et al.* (2003) had evaluated the fuel consumption and opined that the present rate of petrol consumption results in depletion of natural reserves. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels by 2050.

With the oil crisis of the 1970s, ethanol became established as an alternative fuel. Many countries started programs to study and develop fuels in an economic way from available raw materials (Demirbas, 2005). Subramanian *et al*. (2005) reported that the present availability of ethanol in India is 744 million litres which is sufficient only to 5 per cent blending with gasoline and not with diesel. Therefore, need to enhance the availability of ethanol to meet the auto-biofuel requirements.

2.2 Substrates for bioethanol production

Several raw materials are being used for the bioethanol production *viz*. easily fermentable sugary feed stocks (molasses), starchy feed stocks (grains) and feed stocks containing complex sugars in the form of cellulose and hemicelluloses.

2.2.1 Sugar feed stocks

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethanol and other by-products. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose or precursors of glucose are the easiest to convert to ethanol. One example of a sugar feedstock is sugarcane molasses. Other biomass feed stocks rich in sugars (known as saccharides) include sugar beet, sweet sorghum, and various fruits. However, these materials are all in the human food chain and, except for some processing residues, are generally too expensive to use for fuel ethanol production (Doelle and Doelle, 1990 and Yadav *et al.,* 1997).

2.2.2 Starchy feed stocks

Another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials can also be fermented after breaking starch molecules into simple glucose molecules. Examples of starchy materials commonly used around the world for ethanol production include cereal grains, potato, sweet potato, and cassava, but the starch being used is edible. This will lead to loss of valuable food for the purpose of ethanol production (Badger, 2002).

2.2.3 Cellulose

Like sugar materials, starchy materials are also in the human food chain and are thus expensive. Therefore, the next alternative is cellulosic material. Cellulose, a major polysaccharide primarily found in agricultural plant residues, industrial and municipal solid wastes. It is a linear polymer of hydro D-glucose units linked by β- 1, 4 glucosidic bonds. Native cellulose exists in the form of micro fibrils, which are paracrystalline assemblies of several dozen(1 4) β-D glucan chains with hydrogen bonds connected to one another. The cellulose micro fibrils are embedded in a matrix of non cellulosic polysaccharides, mainly hemicelluloses. An important, relatively unusual feature of cellulose is its crystalline structure. The cellulose is synthesized in nature as individual molecules (linear chains of glucosylresidues) which undergo self assembly at the site of biosynthesis (Brown and Saxena, 2000). Approximately 30 individual cellulose molecule are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called micro fibrils, and these are in turn assembled into familiar cellulose fibres. Regardless of their orientation, the chains are stiffened by both intra-chain and inter-chain hydrogen bonds. Adjacent sheets over lie one another and are held together by weak inter-sheet Van der Walls forces; despite the weakness of these interactions, their total effect over the many residues in the elementary fibril is considerable (Lynd *et al*.,2002).

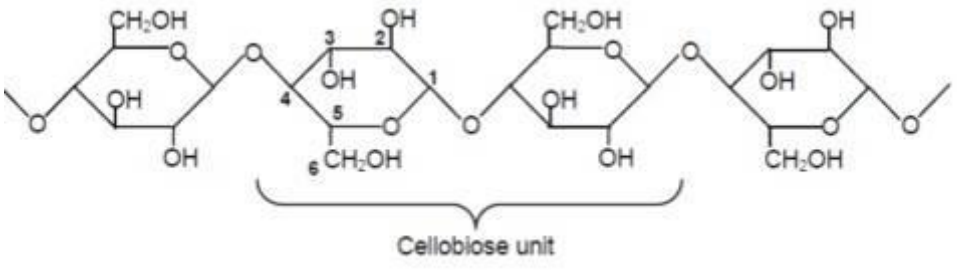


Fig. 1: Schematic illustration of cellulose chain

2.2.4 Hemicellulose

Hemicellulose is a complex, heterogeneous mixture of sugars and sugar derivatives that form a highly branched network. The monomers that comprise hemicellulose are hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose). Some monomers are acetylated (Brigham *et al.,* 1996). Hemicellulose can be classified into three groups, namely, xylans, mannans and galactans based on the polymer backbone that is very often homopolymeric with ß-1, 4 linkage. Xylan is by far the most important component because of its large quantities in the biomass (Hamelinck *et al*., 2005).

Hemicelluloses, the second most abundant polysaccharide in nature, are well suited for ethanol production due to their enormous availability, low cost and environmental benign process. The major fraction in hemeicelluloses is pentosans and the conversion of pentosans to ethanol is problematic. To get the process economized, the conversion of hemicellulose to ethanol with a satisfactory yield is necessary. Significant advances have been made towards the technology of pentosans to ethanol conversion. However, there are technical and economical impediments to the development of commercially viable processes utilizing hemicellulosic derived sugars. Chandel *et al*. (2011).

2.3 Enzymatic degradation of cellulose

The classical scheme for cellulose degradation involves the synergistic action of three classes of enzymes: 1) Endo-1, 4-β-glucanases randomly cleave internal bonds in the cellulose chain. These enzymes may be non-processive or processive (in processive enzymes, enzyme-substrate association is followed by several consecutive cuts in a single polysaccharide chain that is threaded through the active site). 2) Exo-1, 4-β-glucanases attack the reducing or nonreducing end of the cellulose polymer. Processive exo-1, 4-β-glucanases are referred to as cellobiohydrolases; they are among the most abundant components in natural and commercial cellulase mixtures and a subject of intense study. 3) β-glucosidases convert cellobiose, the major product of the endo- and exo-glucanase mixture, to glucose.

These enzymes act synergistically because endo-acting enzymes generate new reducing and non-reducing chain ends for the exo-acting enzymes, which release cellobiose that is converted to glucose by β-glucosidases. It is important to note that natural cellulolytic enzyme systems often contain several exo and endoacting enzymes which may have varying preferences for varying forms of cellulose (crystalline versus amorphous; specific crystal faces). Variation in affinity for the various forms of cellulose may in part be a consequence of variation in the presence of Carbohydrate-Binding Modules (CBMs) that are covalently attached to the catalytic domains of the enzymes.

All these enzymes are hydrolases, i.e. they cleave glycosidic bonds by addition of a water molecule. Commercial cellulase mixtures are mainly based on the cellulolytic enzyme cocktail produced by *H. jecorina* which is dominated by processive cellobiohydrolases (up to 80% of the proteins). Processivity is probably essential to effectively degrade the most crystalline parts of cellulose. It has been pointed out, however, that processive glycoside hydrolases are intrinsically slow and that, therefore, well pretreated cellulose, with more amorphous regions, perhaps could be more efficiently degraded with a cellulase mixture containing less processive enzymes.

2.4 Substrate pre-treatment

There are numerous pre-treatment methods or combinations of pre-treatment methods available. In general, pre-treatment techniques can be grouped into three categories: physical, chemical and biological. Physical pre-treatment methods include comminution, steam explosion and hydrothermolysis. The most common chemical pre-treatment methods used for cellulosic feedstocks are dilute acid, alkaline, organic solvent, ammonia (ammonia fiber explosion AFEX), sulfur dioxide, carbon dioxide or other chemicals to make the biomass more digestible by the enzymes (Schell *et al.,* 2003 and Mosier *et al.,* 2005). Pre-treatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure, so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields.

Effectiveness of ball milling (BM) and wet disk milling (WDM) of sugarcane bagasse and straw was compared and evaluated in terms of enzymatic hydrolysis and fermentation by Ana da Silva *et al*. (2010). At optimum conditions BM-treated bagasse and straw produced glucose of 78.7 and 77.6 per cent and xylose yields of 72.1 and 56.8 per cent, respectively. BM improved the enzymatic hydrolysis by decreasing the crystallinity, while the defibrillation effect favoured enzymatic conversion for WDM samples.

2.4.1 Acid pre-treatment

Concentrated acids such as sulphuric acid (H_2SO_4) and hydrochloric acid (HCl) have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion (Sivers and Zacchi ,1995).

Kuhad *et al*. (2010) have performed acid hydrolysis (3.0%, v/v H_2SO_4, 120°C for 45 min) of *Lantana camara* and observed that the hydrolysis had produced 187.14 mg g-1 total sugars along with fermentation inhibitors such as phenolics (8.2 mg g-1), furfurals (5.1 mg g-1) and hydroxy methyl furfurals (6.7 mg g-1). Later, the toxic compounds from the acid hydrolysate was removed by sequential application of overliming (pH 10.0) and activated charcoal (1.5%, w/v) adsorption. The acid-pretreated biomass of *L. camara* was further delignified through combined pre-treatment of sodium sulphite (5.0% w/v) and sodium chlorite (3.0% w/v) and achieved removal of 87.2 per cent lignin.

2.4.2 Alkali pre -treatment

The alkali pre-treatment is most frequently used to increase the digestibility of some lignocellulose substrates. This treatment was developed as a pulping process to produce high strength, long fibre paper products. Alkali pre-treatment was tried either solely or in combination for efficient delignification and thereby to make the pre-treated substrates accessible for further enzymatic saccharification.

Four commonly used chemical pre-treatment processes namely dilute acid, lime, aqueous ammonia steeping followed by dilute acid hydrolysis, and sodium hydroxide, were evaluated by Chen *et al*. (2009) to provide comparative performance data on corn stover. The pre-treatment of corn stover with 2% NaOH substantially increased the lignin removal and enhanced the accessibility and digestibility of cellulose over other three chemical treatments.

Pre-treatment of switchgrass using aqueous ammonia or NaOH was investigated as a means to enhance the enzymatic digestibility was studied by Gupta and Lee (2010). To increase the effectiveness of pre-treatment, H_2O_2 was supplemented with ammonia or alkali. The composition of pre-treatment liquid indicated that hemicellulose was solubilized during alkaline treatment and existed either in the form of oligomers or as lignin–carbohydrate complex (LCC). LCC formation was prominent in ammonia treatment and in NaOH/H_2O_2 treatment, and that LCC formation protects the hemicelluloses sugars from degradation. Lignin analyses data indicated that NaOH–lignin had more uniform structure and higher O/C ratio than ammonia–lignin and NaOH–lignin had lower aromatic content but higher guaiacyl type structure than ammonia–lignin.

2.5 Saccharification

The hydrolysis of cellulose and hemicelluloses polysaccharides into their respective monomers called as saccharification involves cellulolytic microorganisms or their enzymes namely cellulase and hemicellulase.

2.5.1 Cellulases

Three major types of enzymatic activities are found in cellulase system:

I. Endoglucanases, also called as 1,4 –β- D glucan -4-glucanohydrolaes (EC 3.2.1.4)

II. Exoglucanases, including 1,4-β-D-glucan glucanohydrolases (cellodextrinases) (EC 3.2.1.74), and

III. β –glucosidases or β- glucoside glucohydrolases (EC 3.2.1.21).

Endoglucanases cut, at random, at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends (Bisaria and Ghose, 1981). Exoglucanases act on the reducing or non reducing ends of cellulose polysaccharide chains, liberating either glucose(glucanohydrolases) or cellobiose (cellobiohydrolase) as their major products. Exoglucanases can also act on microcrystalline structure. The β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose. Cellulases are distinguishes from other glycoside hydrolases by their ability to hydrolyse β-1, 4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1-4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton doner and base. The endoglucanase and exoglucanase activities of cellulases are extracellular where as β –glucosidase activity is cell associated where cellobiose must diffuse through the cell mass to be converted into glucose (Demain *et al*., 2005)

2.6 Metabolic pathway

Pathway for sucrose utilisation by yeast

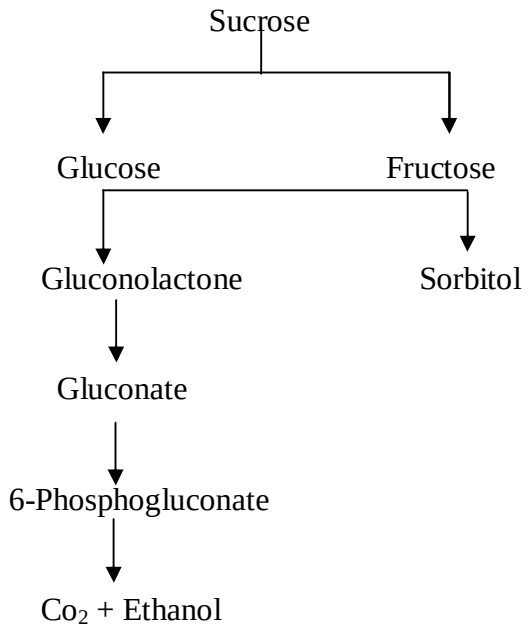


Fig. 2: Metabolic pathway for sucrose utilization by yeast

Metabolic pathways for xylose utilisation by yeast:

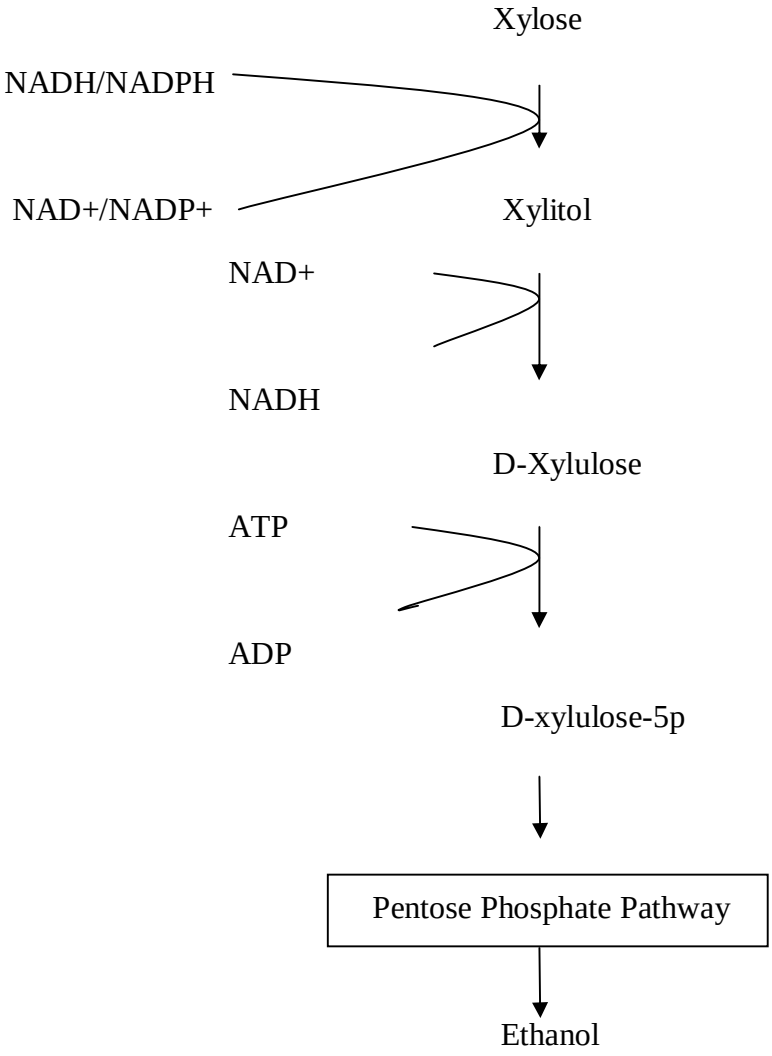


Fig. 3: Metabolic pathway for xylose utilization by yeast

2.7 Hydrolysis and fermentation as two step processes

The fermentation of yeast and enzymatic hydrolysates of *Prosopis juliflora* (Mesquite), containing 18.24g L^{-1} and 18.52g L^{-1} sugars was studied by Gupta *et al*. (2009) using *Pichia stipitis* and *S. cerevisiae*. The fermentation produced 7.13g L^{-1} and 18.52g L^{-1} of ethanol with corresponding yield of 0.39g g^{-1} and 0.49g g^{-1}, respectively from *Pichia stipitis* and *S. cerevisiae.*

Nikolic *et al*. (2010) studied the ethanol fermentation of enzymatically obtained corn meal hydrolyzates by free and immobilized *S. cerevisiae var. ellipsoideus* yeast in a batch system. It was found that the immobilized cell system was superior to the free cell system for higher ethanol tolerance and productivity and lower substrate inhibition, initial glucose concentration of 150 and 176 g L^{-1} for free and immobilized system, initial inoculum concentration of 2% (v/v) and fermentation time of 38 h were the optimum conditions determined for both immobilized and free yeasts.

Shrestha *et al*., (2010) obtained 7.7g ethanol 100 g^{-1} corn fiber which was pre-treated with 2 per cent NaOH (w/w) at 30^0C for 2 h followed by microbial saccharification and saccharification with commercial cellulase enzyme- Spezyme-CP. They reported that solid- fermentation of corn fiber with fungi reduced the dosage of commercial cellulase enzymes during SSF.

2.8 Simultaneous Saccharification and Fermentation (SSF)

The fermentation characteristics of *S. cerevisiae* strain 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 *S. cerevisiae* VSJI, for simultaneous saccharification and fermentation of grains and obtained ethanol yield of 2.90 per cent (v/v).

Choi *et al*. (2010) performed simultaneous saccharification and fermentation of liquefied cassava (*Manihot esculenta*) starch in a 5 L lab-scale jar fermenter at 32 °C for 66 h with an agitation speed of 2 Hz using two ethanol-producing novel strains of *S. cerevisiae*, CHY1011 and CHFY0901. Under these conditions, *S. cerevisiae* CHY1011 and CHFY0901 yielded a final ethanol concentration of 89.1 ± 0.87g L^{-1} and 83.8 ± 1.11g L^{-1}, a maximum ethanol productivity of 2.10 ± 0.02 g L^{-1} h^{-1} and 1.88 ± 0.01 g L^{-1} h^{-1}, and a theoretical yield of 93.5 ± 1.4%and 91.3 ± 1.1%, respectively.

Shen and Agblevor, (2010) studied the kinetics of the simultaneous saccharification and fermentation (SSF) of cellulose to ethanol using a highly refined standard cellulose (microcrystalline cellulose, (Avicel PH101), with a prior hydrolysis phase (semi-simultaneous saccharification and fermentation (SSF). They observed that the 24-h pre-hydrolysis + 48-h SSF (SSF 24) produced higher yield and higher productivity of ethanol than other models.

For the continuous operation in the SSF, the productivity of SSF 24 was much higher than that of SSF 12 though the ethanol concentrations of both cases did not differ significantly.

Simultaneous saccharification and fermentation experiments carried out with optimised pre-treatment conditions resulted in ethanol yields of 163 g kg^{-1} ensiled hemp (dry matter) (71% of the theoretical maximum) and 171 g kg^{-1} dry hemp (74%), which corresponds to 206–216 l Mg^{-1} ethanol based on initial dry material from the dry and ensiled industrial hemp (*Cannabis sativa* L.), which was steam impregnated with 2% SO_2 followed by steam pre-treatment at 210° C for 5 min (Sipos *et al.,* 2010).

2.9 Co-fermentation

The production of ethanol from starch by a co-immobilized mixed culture system of aerobic and anaerobic microorganisms such as a *Aspregillus awamorii* and *Zymomonas mobilis* was investigated by Tanaka *et al.* (1986) and got 25 g ethanol form 100 g L^{-1} of starch.

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 grain 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 in 48 hours. Co-fermentation of glucose and xylose with immobilized *P. stipitis* and *S. cerevisiae* was studied by Grootjen *et al.* (1990) and glucose conversion was found to be 0.13 g g-1 h-1. Czarnecki and Grajek (1991) studied the influence of temperature and incubation time of 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. Today most fuel ethanol is produced by either the dry grind or wet mill process. Current technologies allows for 2.5 gallons (wet milling process) to 2.8 gallons (dry milling process) of ethanol per bushels of corn. (Bothast and Sclicher, 2005).

Co-immobilized cultures of *S. cerevisiae* and *C. shehatae* which convert glucose and xylose simultaneously to ethanol yield of 0.48 g^{-1} total sugars was studied by Lebeau *et al*., (1997).

Srivastava *et al.,* (1997) used three isolate (isolates 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.

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}).

Shafaghat *et al*. (2011) reported ethanol production from molasses, dates and sugarcane syrup with sugar concentration of 35 g/L in batch culture retention time, 24 h; initial sugar concentration100 g L^{-1} exhibited highest ethanol (45.5 g L^{-1}, yield 89%) in a packed bad reacter loaded with immobilized *S. cerevisiae* beads (3mm). After 16h of cultivation free cells were harvested for immobilization. Entrapment method was used to encapsulate free cells using 2.5 per cent sodium alginate solution, solidified in calcium chloride bath. Growth kinetic model for *S. cerevisiae* in batch culture and rate model for performance of immobolized cell reactor were determined.

Park *et al*. (2012) reported ethanol production from cellulose was carried out in one-pot bioethanol production process with *A. cellulolyticus* C-1 and *S. cerevisiae*. Cellulase producing-medium supplemented with 2.5 g L^{-1} of yeast extract was used for productions of both cellulase and ethanol. Cellulase production was achieved by *A. cellulolyticus* C-1 using Solka-Floc (SF) as a cellulase-inducing substrate. Subsequently, ethanol was produced with addition of both 10% (v/v) of *S. cerevisiae* inoculum and SF at the culture time of 60 h. Dissolved oxygen levels were adjusted at higher than 20 per cent during cellulase producing phase and at lower than 10% during ethanol producing phase. Cellulase activity remained 8–12 FPU/ml throughout the one-pot process. When 50–300 g SF/l was used in 500 ml Erlenmeyer flask scale, the ethanol concentration and yield based on initial SF were as 8.7–46.3 g L^{-1} and 0.15–0.18 (g ethanol/g SF), respectively. In 3L fermentor with 50–300 g SF/l, the ethanol concentration and yield were 9.5–35.1 g L^{-1} with their yields of 0.12–0.19 (g g^{-1}) respectively.

2.10 Yeast strains for bioethanol production

Lee *et al*. (1986) reported that yeasts able to grow on D-xylose were screened for the ability to hydrolyze xylan. A total of only 19 of more than 250 strains yielded a positive test by xylanase activity. The activity was localized largely in the genus *Cryptococcus* and in *Pichia stipitis* and its anamorph *Candida shehatae*. The ability to hydrolyze xylan was generally uncoupled from that to hydrolyze cellulose; only three of the xylan-positive strains also yielded a positive test for cellulolytic activity. Of the 19 xylanolytic strains, 2, *P. stipitis* CBS 5773 and CBS 5775, converted xylan into ethanol, with about 60 per cent of a theoretical yield computed on the basis of the amount of D-xylose present originally that could be released by acid hydrolysis.

Microbial production of cellulase and bioethanol were studied using Sugar Beet Pulp (SBP) as a substrate by *Trichoderma reesei* and *S. cerevisiae*, respectively. The subculture medium for cellulase production was a salt solution. Fungal cells were sub-cultured in an orbital shaker at 30°C for 4 to 6 days and then were used as an inoculum. Exponential cells were inoculated into a medium containing SBP. A maximum cellulase activity of 0.46 IU/ml of filter paper activity was obtained. Ethanol yield was higher in pretreated SBP than in non-treated SBP. After 24 h fermentation period, 0.11 g L^{-1} ethanol was produced from pretreated SBP, while non-treated SBP produced 0.05 g L^{-1} ethanol (Moosavi-Nasab and Majdi- Nasab, 2010)

Shuhei *et al*. (2010) demonstrated direct ethanol production from amorphous cellulose using cellulase expressing yeast. Cellulase displaying yeast more efficiently produced ethanol from phosphatic acid swollen cellulose. All enzymes were successfully expressed on the cell surface or in culture supernatants in their active form and cellulose degradation was increased 3 to 5 fold by co expressing endogluconases and cellobiohydrolases. This result showed that cell surface display is more suitable for direct ethanol fermentation from cellulose.

Yanase *et. al. (*2010) demonstrated that direct ethanol fermentation from amorphous cellulose using cellulase-coexpressing yeast. Endoglucanases (EG) and cellobiohydrolases (CBH) from *Trichoderma reesei*,and β-glucosidases (BGL) from *Aspergillus aculeatus* were integrated into genomes of the yeast strain *S. cerevisiae* MT8-1. BGL was displayed on the yeast cell surface and both EG and CBH were secreted or displayed on the cell surface. Direct ethanol fermentation from 10 g/L phosphoric acid swollen cellulose (PASC) was also carried out using EG-, CBH-, and BGL-co-expressing yeast. The ethanol yield was 2.1 g/L for EG-, CBH-, and BGL-displaying yeast, which was higher than that of EG- and CBH-secreting yeast (1.6 g L^{-1} ethanol). This results showed that cell surface display is more suitable for direct ethanol fermentation from cellulose.

Sangkharak *et. al. (*2011) isolated Five novel cellulase-producing bacteria and identified through 16S rRNA sequence as *Cellulomonas* sp. The activity of enzymes (mainly xylanase and endoglucanase) produced from isolated strains was almost present extracellularly and the production of enzyme was dependent on cellulosic substrate (xylan, rice straw and waste paper) used for growth. The optimal condition for cellulase production consisted of 4% of wastepaper with controlled pH at 6 and cultivation temperature at 35^{o}C. *Cellulomonas* sp. strain TSU-03 produced the highest activity of xylanase and endoglucanase at 1860.1 and 388.5 U mg^{-1} protein, respectively. At 50^{o}C, cellulase was highly stable and losing less than 20% of initial activity after 24 h of incubation.Therefore, waste paper hydrolysate was utilized as substrate for ethanol production using *S. cerevisiae*, the highest ethanol production was 12.5 g L^{-1} after 48 h of cultivation under separate hydrolysis and fermentation (SHF) process.

Rai *et. al*. (2012) investigated production of cellulase in low cost medium by thermotolerant yeast. After screening, an efficient yeast isolate having capability of C1 (exo-gluconase) and Cx (endo-gluconase) production was isolated and designated as strain R-1. Maximum enzyme production was achieved at 50°C, pH 5.5 in the medium containing bagasse powder 4% (w/v), and ammonium sulphate 0.1% (w/v) after 72 hours of incubation. The composition containing bagasse powder, 4% (w/v); ammonium sulphate, 0.5 % (w/v); and glucose, 0.5% (w/v) achieved better production after complete medium optimization. The isolated yeast was able to produce C1 (exo-gluconase) and Cx (endo-gluconase) enzymes in appropriate concentrations on a crude cellulosic substrate.

2.11 Engineering of cellulolytic yeast

The yeast strains capable of producing cellulolytic enzymes, methods for construction of these strains, recombinant DNA vectors needed in the construction of these strains, methods used in the construction of these vectors, and cDNA copies of cellulolytic enzymes coding genes.

Chromosomal genes coding for three different cellulases, CBH I, CBH II and ENDO II were isolated from a λ phage gene library of *T. reesei* by differential hybridisation. Fragments of these genes were used to isolate full length cDNAs from a *T. reesei* cDNA library. cDNAs for the three cellulases CBH I, CBH II and ENDO II and the CBH I gene were transferred to suitable 2 μ yeast plasmids. The cellulases produced by the yeast were shown to have similar activities to the native fungal enzyme. A cellulolytic yeast strain *S. cerevisiae* VTT-RC-84001 produced in accordance with the present invention has been deposited in the National Collection of Yeast Cultures, Norwich, United Kingdom, under the deposit number NCYC No. R 128. (Knowles *et al*., 1990)

For direct and efficient ethanol production from cellulosic materials, Fujita *et al*. (2002) constructed a novel cellulose degrading yeast strain by genetically displaying two cellulolytic enzymes on the cell surface of *S. cerevisiae*. By using a cell surface engineering system based on agglutinin, endoglucanase II (EGII) from the filamentous fungus *Trichoderma reesei* QM9414 was displayed on the cell surface as a fusion protein containing an RGSHis6 (Arg-Gly-Ser-His6) peptide tag in the N-terminal region. Localization of the RGSHis6–EGII agglutinin fusion protein on the cell surface was confirmed by immunofluorescence microscopy.

The yeast strain displaying EGII showed significantly elevated hydrolytic activity toward barley glucan, a linear polysaccharide composed of an average of 1,200 glucose residues. In a further step, EGII and glucosidase 1 from *Aspergillus aculeatus* No. F-50 were codisplayed on the cell surface.

The resulting yeast cells could grow in synthetic medium containing glucan as the sole carbon source and could directly ferment 45 g of glucan per litre to produce 16.5 g of ethanol per litre within about 50 h. The yield in terms of grams of ethanol produced per gram of carbohydrate utilized was 0.48 g g^{-1}, which corresponds to 93.3% of the theoretical yield.

Wiedermann and Keller (2006) reported that hydrolysates of lignocellulosic materials contain hexose and pentose sugars. The yeast *S. cerevisiae* is optimal use of industrial ethanol production, however it cannot metabolise the pentose D-Xylose and L-arabinose. Several genetic engineering strategies have been used in attempts to facilitate xylose or arabinose by *S. cerevisiae*. Xylose fermentation has been achieved either by expressing xylose reductases and xylitol dehydrogenases from fungal sources or by expressing xylose isomerases. Arabinose utilization in *S. cerevisiae* has been achieved by expressing bacterial or fungal pathways.

Industrial strains of the yeast *S. cerevisiae* are widely used for the large scale production of fuel ethanol. Henck *et al*. (2010) constructed a genetically manipulable industrial *S. cerevisiae* strain. The strain can be easily transformed with exogenous DNA, exist as stable haploids of both mating types, efficiently mates and forms viable diploids. Diploids have very high sporulation effiency and produce viable spores. This strain has high tolerance to alcohol, a large range of fermentation temperatures and grows rapidly to high cell density. Several cellulase genes from different families were integrated into genome of the strain. High throughput screening of several thousand integrants allowed identification of strains with the highest cellulolytic activities. These cellulolytic yeast strains ferment pretreated cellulose to ethanol with yields of 65% of theoretical in 48 hours.

Wen *et al*. (2010) reported that combining with cellulase production, cellulose hydrolysis, and sugar fermentation into a single step, consolidated bioprocessing (CBP) represents a promising technology for biofuel production. Engineering of *S. cerevisiae* strains displaying a series of uni, bi, and trifunctional minicellulosomes consist of (i) a miniscaffoldin containing a cellulose-binding domain and three cohesion modules, which was tethered to the cell surface through the yeast a-agglutinin adhesion receptor, and (ii) up to three types of cellulases, an endoglucanase, a cellobiohydrolase, and a glucosidase, each bearing a C-terminal dockerin. Compared to the unifunctional and bifunctional minicellulosomes, the quaternary trifunctional complexes showed enhanced enzyme-enzyme synergy and enzyme proximity synergy. More importantly, surface display of the trifunctional minicellulosomes gave yeast cells the ability to simultaneously break down and ferment phosphoric acid-swollen cellulose to ethanol with a titer of 1.8 g l^{-1}.

Chang *et al*., (2013) achieved an economical cellulosic ethanol production, a host that can do both cellulosic saccharification and ethanol fermentation is desirable. Promoter-based Gene Assembly and Simultaneous Over expression (PGASO) was applied to engineer *Kluyveromyces marxianus* KY3, which is a thermo and toxintolerant yeast. A recombinant strain, called KR5, that is capable of simultaneously expressing exoglucanase and endoglucanase (both of *Trichoderma reesei*), a β glucosidase (from a cow rumen fungus), a neomycin phosphotransferase, and a green fluorescent protein. High transformation efficiency and accuracy were achieved as ~63% of the transformants was confirmed to be correct. KR5 can utilize beta-glycan, cellobiose or CMC as the sole carbon source for growth and can directly convert cellobiose and beta-glycan to ethanol.

Thus multi-gene assembly in a single step in a yeast species was successfully engineered with a five-gene cassette assembly and the new host is capable of co-expressing three types of cellulase genes.

Consolidated bioprocessing (CBP), combining cellulase production, saccharification, and fermentation into one step, has been proposed as the most efficient way to reduce the production cost of cellulosic bioethanol. Kim *et al*. (2013) developed a cellulolytic yeast consortium for CBP, based on the surface display of cellulosome structure, mimicking the cellulolytic bacterium, *Clostridium thermocellum.* Designed cellulolytic yeast consortium composed of four different yeast strains capable of either displaying a scaffoldin (mini CipA) containing three cohesin domains derived from *C. thermocellum*, or secreting one of the three types of cellulases, *C. thermocellum* CelA (endoglucanase) containing its own dockerin, *Trichoderma reesei* CBHII (exoglucanase) fused with an exogenous dockerin from *C. thermocellu* or *Aspergillus aculeatus* BGLI (β-glucosidase). The secreted dockerin-containing enzymes, CelA and CBHI were randomly assembled to the surface-displayed mini CipA via cohesin-dockerin interactions.

A mixture of cells with the optimized mini CipA: CelA: CBHII:BGLI ratio of 2:3:3:0.53 produced 1.80 g L^{-1} ethanol after 94 h, indicating about 20% increase compared with a consortium composed of an equal amount of each cell type (1.48 g L^{-1}).

2.12 Saccharification by microorganisms

Jeya *et al*. (2010) reported that *Agaricus arvensis* a new basidiomycrtous fungus produced the highest endogluconase (EG), Cellobiohydrolase (CBH), and β-glucosidase(BGL) activities of 0.3, 3.2, 8 υ per mg- protein respectively, with rice straw as the carbon source. Further, saccharification of the woody biomass (poplar) with *A. arvensis* cellulase as the enzyme source released a high level of fermentable sugars A total reducing sugar level of 29g L (293 mg g^{-1} substrate) was obtained at an enzyme concentration of 65 FPU g^{-1} substrate after optimization of the hydrolysis parameters.

Qing *et al*. (2010) reported that xylose, xylan and xylo-oligomers dramatically decrease conversion rates and yields of saccharification. Xylooligomers were more inhibitory than xylan or xylose in terms of a decreased initial hydrolysis rate and a lower final glucose yield even for a low concentration of 1.67 mg ml^{-1}. At a higher concentration of 1.25 mg ml^{-1}, xylooligomers lowered initial hydrolysis rates of Avicel by 82 per cent and the final hydrolysis yield by 38 per cent. A comparison among glucose sugars and xylose sugars also showed that xylooligomers were more powerful inhibitors than well- established glucose and cellobiose. Typically, the enzymatic hydrolysis rate of lignocellulosic biomass is fast initially but then slows down more rapidly than can be explained by just consumption of substrate. Although several factors including enzyme inhibition, enzyme deactivation, a drop in substrate reactivity or non productive binding of enzyme to lignin could be responsible for this loss of effectiveness.

2.13 Yeast strains exhibiting cellulolytic activity

Yeast, specifically the *Saccharomyces* strains, are facultative anaerobic organisms capable of producing ethanol. Yeast metabolize most sugars through the glycolytic pathway to produce energy and necessary growth intermediates (Ingeldew 1999). To limit high concentrations of acidic end products synthesized in glycolysis, such as pyruvic acid and acetic acid, yeast convert these acids to ethanol and carbon dioxide. Although yeast are not very tolerant of acidic products, they are very tolerant of ethanol. *S. cerevisiae* are able to produce up to 51.1% weight ethanol/weight glucose from glucose derived from starch (Ingledew 1999).

Bajaj *et al*. (2001) screened twenty two strains of yeast, isolated from natural sources for ethanol production, of these 5 isolates were found to be produced 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 natural characters. Two isolates SBS13 and SBS14 were found to be of desired properties for exploiting at commercial level.

Devi and Shankar (2009) reported that the yeast species with cellulose activity was isolated from the soil using standard procedure. Two isolates were found to produce relatively high amount of cellulose. The production of cellulose by the two isolate was achieved in liquid shake culture containing Carboxymethyl cellulose as substrate. Ethanol production was checked in the media containing CMC as substrate after saccharification and fermentation by *S. cerevisiae* cells the ethanol yield was found to be maximum on the 9^{th} day culture of both the isolates. Thus the current work deals with comparison of cellulose activity of the two isolates and alcohol production of two isolates by simultaneous saccharification and fermentation.

Mangunwardoyo *et al*. (2011) isolated a total of 245 yeast from Gunung Halimun National Park (GHNP) which were screened for cellulolytic activity using 0.2% cellulose-azure. The 16 isolates having cellulolytic activity were further screened for carboxymethyl cellulase (CMCase), avicelase and cellobiase using specific substrates (carboxymethyl cellulose, avicel and cellobiose) with Teather and Wood method. The results showed that 7 isolates have CMCase; 6 isolates have cellobiase; 2 isolates have CMCase and cellobiase; and 1 isolate has CMCase and avicelase and cellobiase activities. Isolate S 4121 has the highest CMCase activity and identified as *Trichosporon sporotrichoides* (van Oorschot) van Oorschot and de Hoog UICC Y-286.

Seven cellulose degrading bacteria bacterial strains were isolated and screened for cellulase production in submerged fermentation process. Among these seven tested bacterial strains; ASN2 showed maximum yield for cellulase production which was further characterized by biochemical and morphological tests and identified as *Cellulomonas* sp.

ASN2. Supplementation of glucose, peptone and cysteine to the fermentation medium are favoured enzyme secretion. The optimum pH and temperature for the activity of crude enzyme was 7.5 and 60^{o}C, respectively. Metal profile of the enzyme indicated that Co^{2+} and Mn^{2+} are the strong stimulators while Hg^{2+} and Fe^{2+} inhibited the activity of cellulase from cellulolytic bacterial strain, *Cellulomonas* sp.ASN2.

It indicated that favourable fermentation conditions and the selection of a suitable growth medium played a key role in the production of cellulase from newly isolated *Cellulomonas* sp. ASN2 (Irfan *et al.,* 2012).

2.14 Type of fermentors for ethanol production

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 sugar (Mohammad *et al.,* 1999). Krishana *et al. (*2000) studied the economics of fuel ethanol production from dry milled corn starch in fluidized bed reacter (FBRs) using immobilized biocatalysts.

Ethanol production on a pilot scale for the conversion of high solid 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^{0}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.An ethanol yield of 86 per cent (w/w), reaching 50 g dm^{-3} in a bioreactor.

2.15 Pilot scale production of bioethanol

Ballerini *et al*. (1994) studied large scale ethanol production. They performed hydrolysis of the steam exploded poplar in 25 M3 stirred reactor using cellulase enzyme of *Trichoderme reesei*. The pre-treated substrate at 5-6% feed stock concentration when incubated for 72 h at 50^{0}C and 4.8 pH with16 FPU g-1 substrate produced 96.2 per cent hydrolysis of polysaccharide. The hydrolysate had 93.6 per cent glucose, 3.5 per cent cellobiose 2.9 per cent xylose. Upon fermentation with *S. cereviseae* ATCC 26603 produced 160-190 kg ethanol from 1000 kg poplar wood.

Sharma *et al*. (2004) observed maximum ethanol content of 0.449 (44.9%) and 0.446 (44.6%) g g^{-1}, respectively from sunflower hulls in scaled up fermentation of 1 L and 15 L under optimum conditions.

MATERIAL AND METHODS

The ethanol production is directly dependent on the availability of molasses and the output varies according to the annual production of sugarcane. The recent policy on decontrolling molasses in India might cause not only the short supply of the commodity but also hike in its price. To overcome the shortage, alcohol is some time imported. The shortfall in ethanol production can also be met by utilizing alternative renewable sources like sugar, starch rich substrates and cellulosic substrates for economic reasons. Sugar rich substrates are comparatively expensive, but it could be easily and readily employed for ethanol production.

On the other hand, though cellulosic biomass is cheaper, their conversion to ethanol is successful following two step process; hydrolysis and fermentation. In order to reduce the step present study was conducted to know the role of yeast in degradation of cellulose. The investigation was carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad. The details of materials used and methods employed during the course of investigation are presented in this chapter.

3.1 Isolation of yeast strains for cellulolytic activity

Substrate to be used for isolating yeast strains:

Sl. No.	Substrates	Source	Location(GPS reading)
1	Straw berry	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
2	Custard apple	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m
3	Jack fruit	Field of Yettinagudda village, Dharwad	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m
4	Ber	AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m
5	Apple	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
6	Sugarcane	Dharwad city market	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m
7	Molasses	Distillery unit, Dharwad	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m
8	Sweet lemon skin	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
9	Grapes	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
10	Lemon pulp	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
11	Sapota	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
12	Pineapple	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m
13	Rotten banana peel	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0

			Ele 716m
14	Cactus flower	Behind guest house, AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m
15	Over matured paragrass	Behind Wheat scheme, AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m
16	Forest leaf litter,	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m
17	Composted plant residues	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m

The samples were selected based on the cellulosic nature of the outer skin of the degraded substrates and other sources which are partially composted. For e.g. rotten fruit rinds, forest leaf litter, compost, bagasse, composted plant residues, cactus flower, fruit samples, food stuff, over matured paragrass and FYM etc. were chosen for isolation.

3.2 Isolation

The isolations were made by following standard serial dilution technique using normal MGYP media (Appendix –I) and incubated for 24-48 h at 37^0 C. Based on the colony morphology, typical yeast colonies were chosen, purified and observed under the microscope (methylene blue staining) for their budding character and cell morphology.

3.3 Maintenance of the culture

The identified potential isolates used for the investigation were maintained on MGYP Agar (Malt Extract Glucose Yeast Extract Peptone) medium or CMC (Carboxy Methyl Cellulose) agar medium (Appendix-I).

3.4 Screening of the strains for cellulolytic activity (Teather and Wood, 1982)

All the 104 isolates were spotted individually on media containing carboxy methyl cellulose as sole source of carbon and incubated at 37^0 C for 48 h.

The plates were flooded with 0.01 % of congo red stain for 15 min followed by destaining with 1M sodium chloride solution for 15 min. The clear zone around the colonies indicated the hydrolysis of cellulose by yeast.

The isolates were tested for the zone of hydrolysis and measured in terms of mm. The individual isolates were evaluated in terms of their ability to hydrolyse cellulose quantitatively by estimating reducing sugars at 5 days interval up to 15 days.

3.5 Biochemical characterization of the screened isolates upon various sugars

In order to know the carbon utilization efficiency of the isolates, the isolates were grown on various carbon sources *viz.* glucose, galactose, sucrose, maltose, lactose, starch, D-xylose, arabinose and D-ribose (Ribereau-Gayon *et al*., 2007).

3.6 Performance of yeast isolates on delignified substrates

The selected crop residues (bagasse and paddy straw) were delignified to obtain cellulose. Efficiency of strains were compared with commercial cellulase. The efficiency was evaluated in terms of degradation of cellulose, release of reducing sugars and bioethanol production.

3.6.1 Preparation of substrate for activity

The fresh substrates *viz*., sugarcane bagasse, sugarcane trash, paddy straw were chopped into small pieces, dried at 60^{o}C in hot air oven for 12 h and powered by dry milling to obtain particle size of 500 µ.

3.6.2 Chemical treatment using alkali

Ten grams of the oven dried substrates were transferred separately into 250 ml Erlenmeyer flasks. All the substrates were treated separately with 50 ml of 2.5 and 3 per cent NaOH (w/v) separately and autoclaved for 6 h and 8 h. The contents were drained and throughly washed to remove alkali. The pulp obtained was used for estimation of cellulose. The alkali was sufficient enough to moisten entire substrate except in case of bagasse where additional 10 ml was used.

3.7 Enzyme assay (T. K. Ghose, 1987)

Enzyme assays such as FPase activity and CMCase activity were performed to know saccharification potential of the strains and commercial enzymes used during the investigation on bio-ethanol production. The methods followed are as follows.

3.7.1 FPase activity

Whatman No. 1 filter paper (50 mg) was taken in a test tube and 0.80 ml of sodium citrate buffer 0.05M, pH 4.8 and 0.80 ml of appropriately diluted enzyme or enzyme extract were added. The reaction mixture was incubated for 10 min at 50ºC. After the incubation, 2.4 ml of DNSA was added and tubes were kept under room temperature for 15 min so as to lower the temperature of reaction mixture to room temperature. Appropriate enzyme blank and reagent was also included in the estimations. The reducing sugars were estimated by following DNSA method. The enzyme activity was expressed in terms of filter paper units (FPU). One unit of filter paper activity was defined as the amount of enzyme required to liberate one µ mole reducing sugar (expressed as glucose equivalents) in one minute under reaction condition. The enzyme activity was calculated by using the following formula.

$$\text{Enzyme activity (U ml}^{-1}\text{)} = \frac{\text{OD x µg sugar (from standard graph)x dilution factor}}{\text{0.1x Mol. Wt of glucose x incubation time (min) x volume of enzyme(ml)}}$$

3.7.2 CMCase activity (T. M. Wood and K. M. Bhat, 1988)

Carboxymethyl cellulose (CMC) of 0.50 g was dissolved in 49.50 ml of sodium citrate buffer (0.50 M, pH 4.8) by vigorous stirring until the CMC was completely dissolved. This solution had the cellulose concentration of one mg per ml. To 0.80 ml of CMC solution (1mg ml^{-1}) 0.80 ml of appropriately diluted enzyme or enzyme extract was added. The reaction system was incubated for 10 min at 50^0C .After the incubation 2.4 ml DNSA was added and the tubes were kept under room temperature for 15 min so as to lower the temperature of reaction mixture to room temperature .Appropriate enzyme blank and reagent was also included in the estimations .

The reducing sugars were estimated by following DNSA method. The enzyme CMCase activity was expressed in units. One unit of cellulase activity was defined as the amount of enzyme required to liberate one µ mole of reducing sugar (expressed as glucose equivalents) in one minute under reaction conditions. The enzyme activity was calculated by using the following formula.

$$\text{Enzyme activity (U ml}^{-1}\text{)} = \frac{\text{OD x µg sugar (from standard graph)x dilution factor}}{\text{0.1x Mol. Wt of glucose x incubation time (min) x volume of enzyme(ml)}}$$

3.8 Chemical analysis

3.8.1 Estimation of Cellulose: (Goering and Vansoest, 1975)

The cellulose contents of the raw substrate were estimated by determining Acid detergent fibre (ADF). The cellulose contents were expressed in grams. The details of the method followed are explained below.

3.8.2 Acid detergent fibre

Preparation of Acid detergent solution

One litre of 1N sulphuric acid was prepared and in this solution, 20 g of cetyl trimethyl ammonium bromide was dissolved.

Procedure

Raw or pre-treated substrate of 0.5 g was transferred to a 500 ml refluxing flask. To this, 100 ml of acid detergent solution and 2 ml of decahydronaphthalene were added. This mixture was heated to boiling and the heat was regulated to avoid foaming and refluxed for one hour. After one hour of refluxing, the mixture was cooled and filtered through a previously weighed 50 ml capacity gooch crucible of G-1 grade under suction using vacuum pump. The sample in the crucible was washed with hot water to remove acid followed by two washings with acetone. The crucibles were dried at 100^0C for 8 h in a hot air oven. Finally, the crucibles were cooled in a desiccator and dry weight was recorded. The per cent ADF was calculated by the formula given below.

$$\% \text{ ADF} = \frac{Y - X}{W} \times 100$$

Where,

Y = weight of crucible + ADF

X = weight of empty crucible

W = weight of the sample

3.8.3 Acid detergent lignin:

Preparation of acid

72% H_2SO_4 (w/v)

Concentrated sulphuric acid of 73.5 ml was added to a beaker containing distilled water of 26.5 ml.

Procedure

Crucibles containing ADF (acid detergent fibre) were kept in 100 ml Petri dish and the contents of crucibles were filled with cooled 72% H_2SO_4. The contents were stirred with a glass rod to break lumps of residue if any. As the acid drains out, fresh acid was refilled in to the crucibles and frequent stirring was given. After 3 h of acid digestion along with intermittent stirring, the contents were filtered with hot water by vacuum suction to retain the residue and to remove the used acid. Then the crucibles with residues were dried at 100^0C for 8h. After this, the crucibles were cooled in a desicator and weighed (L). The per cent cellulose contents were calculated by the formula given below.

$$\% \text{ Cellulose} = \frac{Y - L}{W} \times 100$$

Where, Y = weight of ADF + crucible

L = weight of crucible + lignin

W = weight of the sample

3.9 Estimation of reducing sugars

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959).

Preparation of reagent

DNSA: one gram of 3, 5-dinitrosalycylic acid (DNSA), 200 mg of crystalline phenol and 50 mg of sodium sulphite were dissolved in 100 ml of one per cent NaOH and was stored at 40^oC. As the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.

Rochelle salt solution (40%)

It was prepared by dissolving 40 g of potassium sodium tartrate in 100 ml distilled water.

Preparation of stock solution of glucose.

Standard stock solution having the concentration of 1 mg glucose ml^{-1} was prepared by dissolving 100 mg of D-glucose in small amount of distilled water and final volume was made up to 100 ml with distilled water.

Preparation of working standard

About 10 ml of the stock was diluted to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 100 µg glucose ml^{-1}.

Procedure

About 0.5 ml of sample was drawn from every treatment into test tubes. The volume was made up to 3 ml using distilled water. DNSA reagent of 3 ml was added to each sample, mixed well. The reagent blank containing 3 ml of distilled water and 3 ml of DNSA reagent was also prepared. Similarly, standards were also included whose glucose concentration ranged from 10 µg to 100 µg. All tubes *viz.,* samples, standards and blank were kept on boiling water bath for 5 minutes. After this one ml of 40 per cent Rochelle salt solution was added when the reaction mixture was still warm. Then the tubes were cooled. The absorbance in terms of optical density of the standards and sample were read at 510 nm using Systronics UV Spectrophotometer-117. The standard glucose was also prepared similarly with concentration ranging from mg to mg ml-1.

3.10 Estimation of total sugars

The amount of total sugars was estimated by Nelson's modification of Somogyi's method (Nelson, 1944).

Preparation of reagents

1N Hydrochloric acid (1N): About 8.8 ml of concentrated HCl was diluted with distilled water to a final volume of 100 ml in a 100 ml volumetric flask. 0.1N hydrochloric acid: About 0.88 ml of hydrochloric acid was diluted and the volume was made up to 100 ml with distilled water in a volumetric flask. Sodium hydroxide (1N): About 4 g of NaOH was dissolved in little amount of distilled water and the final volume was made up to 100 ml. Phenolphthalein indicator: Phenolphthalein powder of 1g was dissolved in 50 ml of 70 percent alcohol. The final volume was made up to 100 ml with distilled water. Procedure: one ml of representative sample from each treatment and replication was taken in test tubes. One ml of 1 N HCl was added to each tube and placed them in boiling water for 15-20 min. Tubes were cooled and one drop of phenolphthalein indicator was added to each tube. 1 N NaOH was added till pink colour appeared followed by addition of 0.1 N HCl till pink colour disappeared. Finally the contents in each tube were mixed well and volume was made up to 5 ml with distilled water 0.5 ml representative sample taken from these tubes and total sugars was estimated by following DNSA method.

3.11 Estimation of ethanol

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

Preparation of reagents

Potassium dichromate ($K_2Cr_2O_7$) 0.23 N: Potassium dichromate of 34 g was dissolved in 500 ml of distilled water. To this 325 ml of concentrated sulphuric acid was added and the volume was made up to 1000 ml with distilled water.Preparation of ethanol stock solution : It was prepared by mixing 12.6 ml of analytical grade ethanol (789 mg/ml) with little amount of distilled water and making up the volume to 100 ml using distilled water, to obtain concentration of 100 mg ethanol/ml.Procedure: One ml of representative sample from each treatment was transferred to 250 ml round bottom flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-750C. The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagents, which was kept at the receiving end. The distillate containing alcohol was collected till total volume of 45 ml was obtained. The, samples were kept in water bath at 600 for 20 min and were cooled.

The volume was made up to 50 ml with distilled water and optical density was measured at 600 nm using spectrophotometer-117 (M/s. Systronics, India). Similarly, the standard ethanol was also carried out with 20-100 mg concentration of standard ethanol.

3.12 Statistical analysis

The result obtained in the investigation on bioethanol production were statistically analysed using factorial, completely randomised design as described by Panse and Sukhatme (1985).

EXPERIMENTAL RESULT

Ethanol is a liquid biofuel produced from sugar rich biomass. The production of ethanol has gained importance in last few years due to the increased dependency on oil and conventional fuels. Ethanol can be blended up to 20 per cent with diesel or petrol. At present ethanol is produced from molasses, which is a byproduct of sugar industries. The cost of production increases as the demand for molasses has increased. The agricultural crop residues such as paddy straw, wheat straw and bagasse are abundantly available having rich source of sugars.

Hence, in the present study such pretreatment substrates were evaluated for maximum production of ethanol followed by fermentation. The results of experiments conducted during the present investigation in order to find out efficient ethanol producing cellulolytic yeast from various natural habitats are presented here.

4.1 Sample collection from various natural habitats for isolation of yeast sps

In order to isolate the yeast sp., total 17 samples were collected from different locations of North Karnataka from various natural sources *viz.* sugarcane field, forest litter, rotten fruits and compost. While collecting the samples Geographical Positioning System (GPS) was adopted to find out the particular latitude, longitude and elevation of the location (Table 1).

4.1.1 Isolation of yeast sps.

Based upon the budding character, creamish smooth surface, oval and round colonies were picked for further study (plate 1). All the colonies were observed under the microscope and based on the budding character, the colonies were selected, purified and maintained for further studies. (Table 2 and plate 2)

4.2 Screening of yeast isolates expressing cellulolytic activity in terms of cellulose hydrolysis

A total of thirty one isolates were selected and screened on the basis of zone of hydrolysis. (Table 3 and plate 3)

4.3 Cellulolytic activity of the yeast isolates in terms of reducing sugars in CMC broth

Thirty one isolates were screened in CMC broth to know the efficiency of the isolates in terms of releasing reducing sugars (Table 4). There is considerable variation in the initial degradation of CMC among the strains. Strain CY-5, CY-18 and CY-65 showed delayed response towards CMC broth in releasing reducing sugars indicating absence of all three cellulase enzymes in different incubation days.

Out of 31 yeast isolates, 13 isolates viz: CY-13, CY-17, CT-30, CY-33, CY-40, CY-47, CY-48, CY-52, CY-58, CY-59, CY-62, CY-65 CY-68, CY-81 and CY-83 showed the immediate response towards degradation of cellulose indicating possibility of presence of all three cellulase enzymes at different incubation days.

Among all isolates screened for reducing sugars the CY-59 showed the highest reducing sugars (174.03 mg g^{-1}) followed by CY-62 (170.03 mg g^{-1}), CY-81(169.02 mg g^{-1}), CY 52 (167.03 mg g^{-1}) and CY-58 (159.83 mg g^{-1}) and the viscosity of the CMC broth was reduced.

4.4 Cellulolytic activity of the yeast isolates in terms of total sugars in Carboxy Methyl Cellulose (CMC) broth

Among all the 31 isolates, 13 isolates CY-13, CY-17, CY-30, CY-33, CY-40, CY-47, CY-48, CY-52, CY-58, CY-59, CY-62, CY-65 CY-68, CY-81 and CY-83 showed the immediate response towards degradation of cellulose indicating possibility of presence of all three cellulase enzymes in different incubation days.

Among all the isolates, CY-59 showed the highest total sugars (193.05 mg g^{-1}) followed by CY-62 (190.05 mg g^{-1}), CY-81(188.98 mg g^{-1}), CY-52(185.03 mg g^{-1}) and CY-58 (180.04 mg g^{-1}) (Table 5). Further non reducing residual sugars were calibrated in isolates CY-52 (18 mg g^{-1}), CY-58 (20.21 mg g^{-1}), CY-59 (19.02 mg g^{-1}), CY-62 (20.02 mg g^{-1}) and CY-81 (19.96 mg g-1) (Table 6).

Table 1: Details of the sources of samples collected for isolating yeasts

Sl. No	Substrates	Source	Location(GPS reading)	No of yeast isolates isolated
1	Straw berry	Dharwad city market	N15^{0}27'42.3 E75^{0}00'46.0 Ele 716m	7
2	Custard apple	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	8
3	Jack fruit	Field of Yettinagudda village, Dharwad	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m	5
4	Ber	AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	5
5	Apple	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	4
6	Sugarcane	Dharwad city market	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m	6
7	Molasses	Dharwad city market	N15^{0}29'47.34" E74^{0}56'37.82" Ele 610m	6
8	Sweet lemon skin	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	5

9	Grapes	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	8
10	Lemon pulp	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	8
11	Sapota	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	6
12	Pineapple	Dharwad City market	N15^{0}27'42.63" E75^{0}00'46.70" Ele 716m	5
13	Rotten banana peel	Dharwad City market	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	8
14	Cactus flower	Behind guest house, AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	4
15	Over matured paragrass	Behind Wheat scheme, AC, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	5
16	Forest leaf litter,	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	8
17	Composted plant residues	MARS, Dharwad	N14^{0}33'51.7" E74^{0}58'46.4" Ele510m	6
Total isolates				104

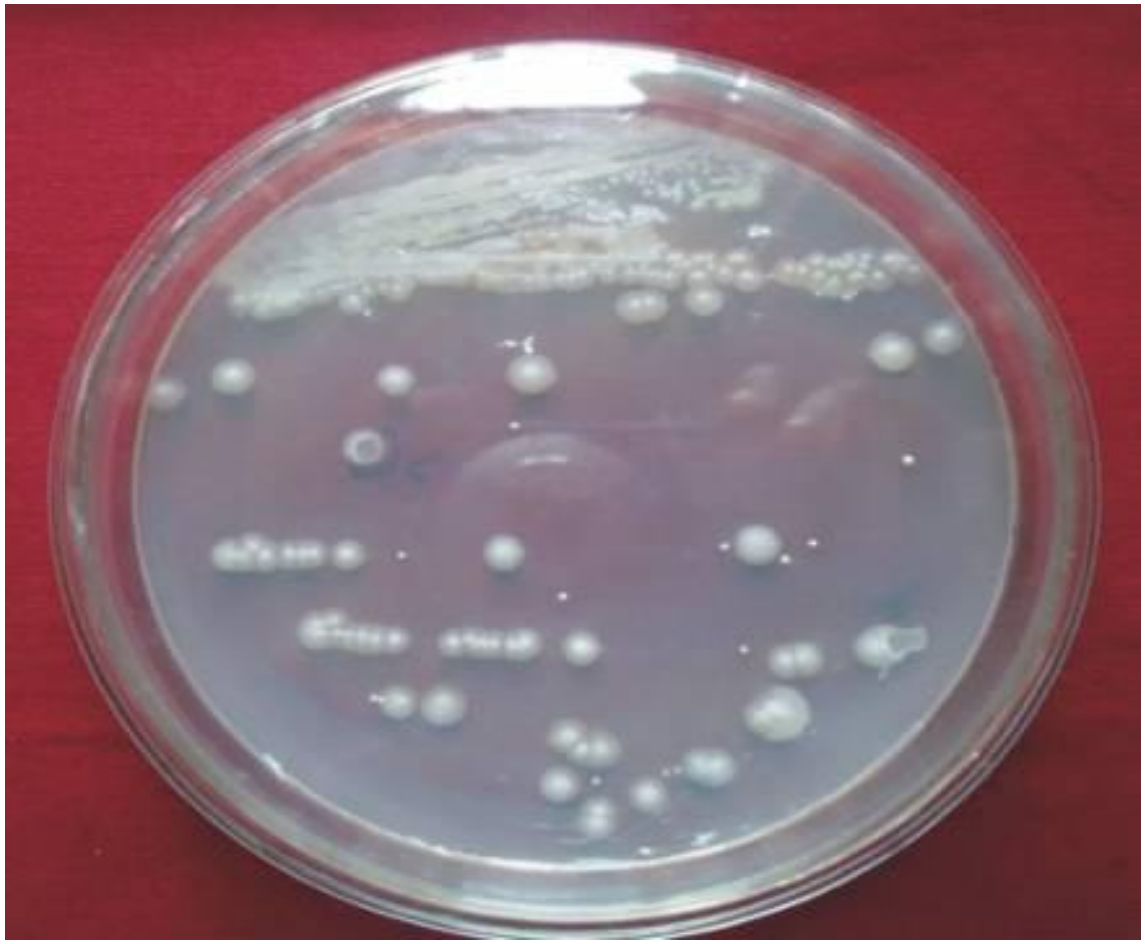


Plate 1: Colonies of yeast isolates on MGYP media

Table 2: Morphological view of the isolated yeasts

Sl. No.	Isolate codes	Colony characteristics	Microscopic observations of cells
1	CY-1 (Straw berry)	Circular, smooth shiny surface	Round cells with budding at one end.
2	CY-2 -do-	Round , smooth shiny surface	Round cells with budding at one end.
3	CY-5 (Sweet lemon skin)	Round, smooth shiny surface	Round cells with budding at one end.
4	CY-7 (Pulp of sweet lemon)	Circular, smooth shiny surface	Oval cells with budding at one end.
5	CY-13 (Papaya)	Round, smooth shiny surface	Round cells with budding at one end.
6	CY-14 (Fermented straw berry)	Round, smooth shiny surface	Round cells with budding at one end.
7	CY-17 (Apple skin)	Round, smooth shiny surface	Oval cells with budding at one end.
8	CY-18 (Pulp of apple)	Circular, smooth shiny surface	Oval, budding of cells
9	CY-20 (Fermented silage)	Round , smooth shiny surface	Round cells with budding at one end.
10	CY-24 (Rotten banana)	Round, smooth shiny surface	Round cells with budding at one end.
11	CY-29 (Skin of pomegranate)	Round, smooth shiny surface	Round cells with budding at one end.
12	CY-30 (Pomegranate)	Circular, smooth shiny surface	Oval cells with budding at one end.
13	CY-31 (pulp of ber fruit)	Round, smooth shiny surface	Round cells with budding at one end.
14	CY-32 (Inner skin of Baer fruit)	Round, smooth shiny surface	Round cells with budding at one end.
15	CY-33 (Lemon pulp)	Round, smooth shiny surface	Oval cells with budding at one end.
16	CY-34 (Custard apple)	Circular, smooth shiny surface	Oval, budding of cells
17	CY-39 (Ber fruit)	Round, smooth shiny surface	Round cells with budding at one end.
18	CY-40 (Skin of pomegranate)	Round, smooth shiny surface	Round cells with budding at one end.
19	CY-42 (Composted plant residue)	Round, smooth shiny surface	Round cells with budding at one end.
20	CY-43 (Custard apple)	Circular, smooth shiny surface	Oval cells with budding at one end.
21	CY-45 (Molasses)	Round, smooth shiny surface	Round cells with budding at one end.
22	CY-47 (Grapes)	Round, smooth shiny surface	Round cells with budding at one end.
23	CY-48 (Straw berry)	Circular, smooth shiny surface	Oval cells with budding at one end.
24	CY-52 (Forest litter)	Circular, smooth shiny surface	Oval, budding of cells
25	CY-58 (Juice of pomegranate)	Round, smooth shiny surface	Round cells with budding at one end.
26	CY-59 (Pulp of sweet lemon)	Circular, smooth shiny surface	Oval cells with budding at one end.
27	CY-62 (Rotten banana peel)	Round, smooth shiny surface	Round cells with budding at one end.
28	CY-65 (Pineapple)	Round, smooth shiny surface	Round cells with budding at one end.
29	CY-68 (Over matured paragrass)	Round, smooth shiny surface	Oval cells with budding at one end.
30	CY-81 (Sugar cane bagasse)	Circular, smooth shiny surface	Oval, budding of cells
31	CY-83 (Cactus flowers)	Round, smooth shiny surface	Round cells with budding at one end.

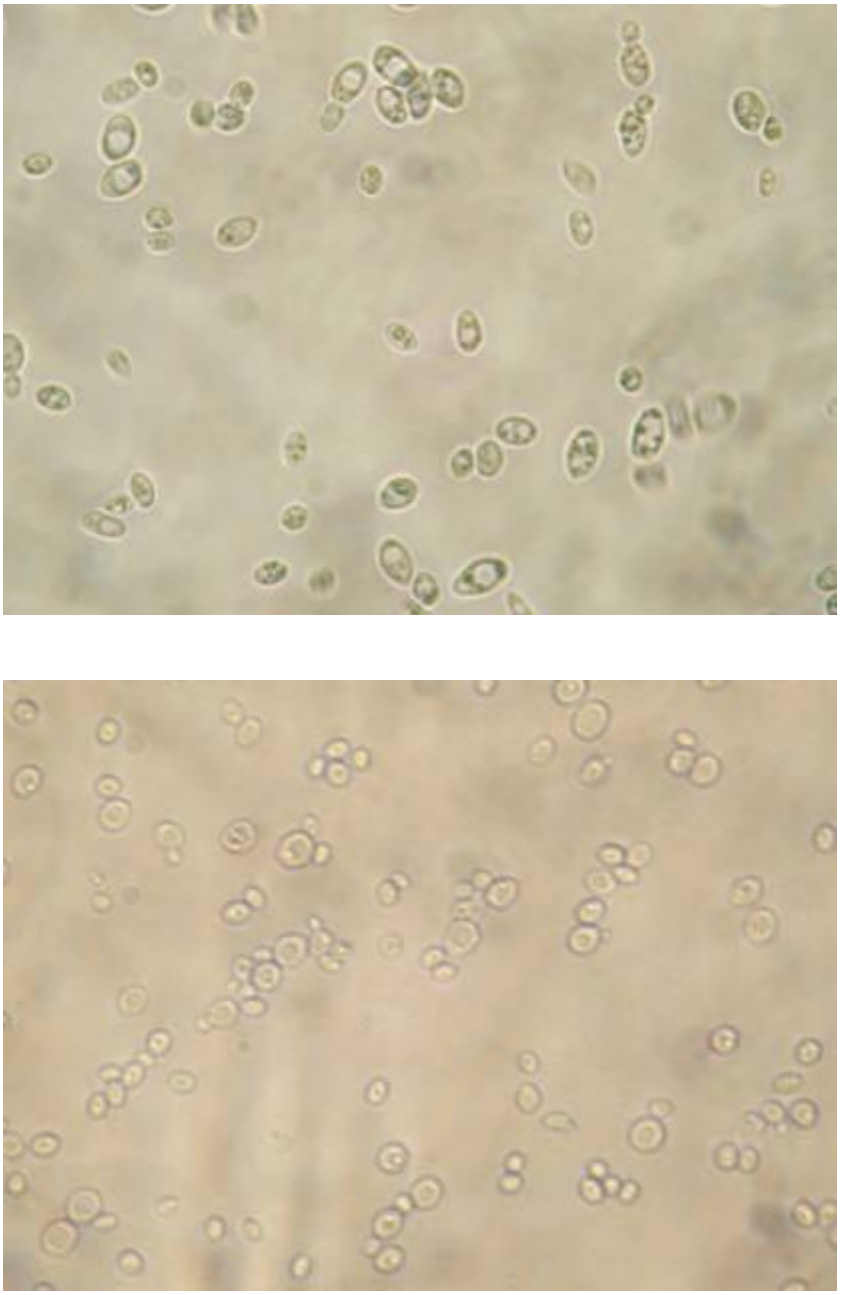


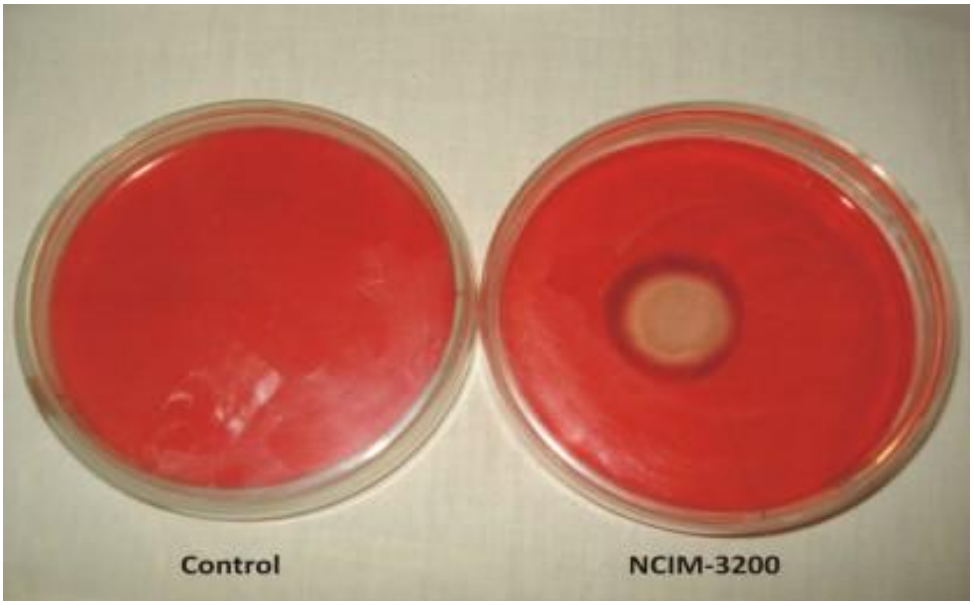
Plate 2: Microscopic view of budding characters of yeast isolates

Table 3: Screening of yeast isolates expressing cellulolytic activity in terms of zone of hydrolysis

Sl No.	Isolate code	Zone of hydrolysis of cellulose in diameter (mm)
1	CY-1 (Straw berry)	13
2	CY-2 -do-	11
3	CY-3 (Apple skin)	0
4	CY-4 (Rotten banana)	0
5	CY-5 (Sweet lemon skin)	9
6	CY-6 (Pomegranate)	0
7	CY-7 (Pulp of sweet lemon)	14
8	CY-8 (Rotten banana)	0
9	CY-9 (Apple skin)	0
10	CY-10 (Pulp of sweet lemon)	0
11	CY-11 (Pulp of sweet lemon)	0
12	CY-12(Apple skin)	0
13	CY-13 (Papaya)	21
14	CY-14 (Fermented straw berry)	13
15	CY-15 (Pomegranate)	0
16	CY-16 (Pomegranate)	0
17	CY-17 (Apple skin)	20
18	CY-18 (Pulp of apple)	13
19	CY-19 (Over matured paragrass)	0
20	CY-20 (Fermented silage)	15
21	CY-21 (Pulp of sweet lemon)	0
22	CY-22 (Over matured paragrass)	0
23	CY-23 (Custard apple)	0
24	CY-24 (Rotten banana)	17
25	CY-25 (Over matured paragrass)	0
26	CY-26 (Pulp of sweet lemon)	0
27	CY-27 (Apple skin)	0
28	CY-28 (Over matured paragrass)	0
29	CY-29 (Skin of pomegranate)	16
30	CY-30 (Pomegranate)	21
31	CY-31 (pulp of baer fruit)	22
32	CY-32 (Inner skin of baer fruit)	19
33	CY-33 (Lemon pulp)	23
34	CY-34 (Custard apple)	16
35	CY-35 (Rotten banana)	0
36	CY-36 (Apple skin)	0
37	CY-37 (Over matured paragrass)	0
38	CY-38 (Pomegranate)	0
39	CY-39 (Ber fruit)	20

40	CY-40 (Skin of pomegranate)	13
41	CY-41 (Custard apple)	0
42	CY-42 (Composted plant residue)	9
43	CY-43 (Custard apple)	16
44	CY-44 (Pomegranate)	0
45	CY-45 (Molasses)	23
46	CY-46 (Pomegranate)	0
47	CY-47 (Grapes)	21
48	CY-48 (Straw berry)	22
49	CY-49 (Rotten banana)	0
50	CY-50 (Apple skin)	0
51	CY-51 (Fermented silage)	0
52	CY-52 (Forest litter)	27
53	CY-53 (Rotten banana)	0
54	CY-54 (Fermented silage)	0
55	CY-55 (Apple skin)	0
56	CY-56 (Over matured paragrass)	0
57	CY-57 (Over matured paragrass)	0
58	CY-58 (Juice of pomegranate)	24
59	CY-59 (Pulp of sweet lemon)	29
60	CY-60 (Custard apple)	0
61	CY-61 (Custard apple)	0
62	CY-62 (Rotten banana peel)	28
63	CY-63 (Rotten banana)	0
64	CY-64 (Custard apple)	0
65	CY-65 (Pineapple)	21
66	CY-66 (Custard apple)	0
67	CY-67 (Custard apple)	0
68	CY-68 (Over matured paragrass)	13
69	CY-69 (Fermented silage)	0
70	CY-70 (Sugar cane bagasse)	0
71	CY-71 (Apple skin)	0
72	CY-72 (Over matured paragrass)	0
73	CY-73 (Sugar cane bagasse)	0
74	CY-74 (Over matured paragrass)	0
75	CY-75 (Pulp of sweet lemon)	0
76	CY-76 (Pulp of sweet lemon)	0
77	CY-77 (Apple skin)	0
78	CY-78 (Sugar cane bagasse)	0
79	CY-79 (Sugar cane bagasse)	0
80	CY-80 (Cactus flowers)	0

81	CY-81 (Sugar cane bagasse)	26
82	CY-82 (Apple skin)	0
83	CY-83 (Cactus flowers)	14
84	CY-84 (Over matured paragrass)	0
85	CY-85 (Cactus flowers)	0
86	CY-86 (Over matured paragrass)	0
87	CY-87 (Cactus flowers)	0
88	CY-88 (Rotten banana)	0
89	CY-89 (Apple skin)	0
90	CY-90 (Cactus flowers)	0
91	CY-91 (Over matured paragrass)	0
92	CY-92 (Rotten banana)	0
93	CY-93 (Pomegranate)	0
94	CY-94 (Apple skin)	0
95	CY-95 (Over matured paragrass)	0
96	CY-96 (Pomegranate)	0
97	CY-97 (Over matured paragrass)	0
98	CY-98 (Rotten banana)	0
99	CY-99 (Rotten banana)	0
100	CY-100 (Apple skin)	0
101	CY-101 (Pomegranate)	0
102	CY-102 (Cactus flowers)	0
103	CY-103 (Cactus flowers)	0
104	CY-104 (Cactus flowers)	0
105	*Saccharomyces cerevisiae* NCIM-3200	31



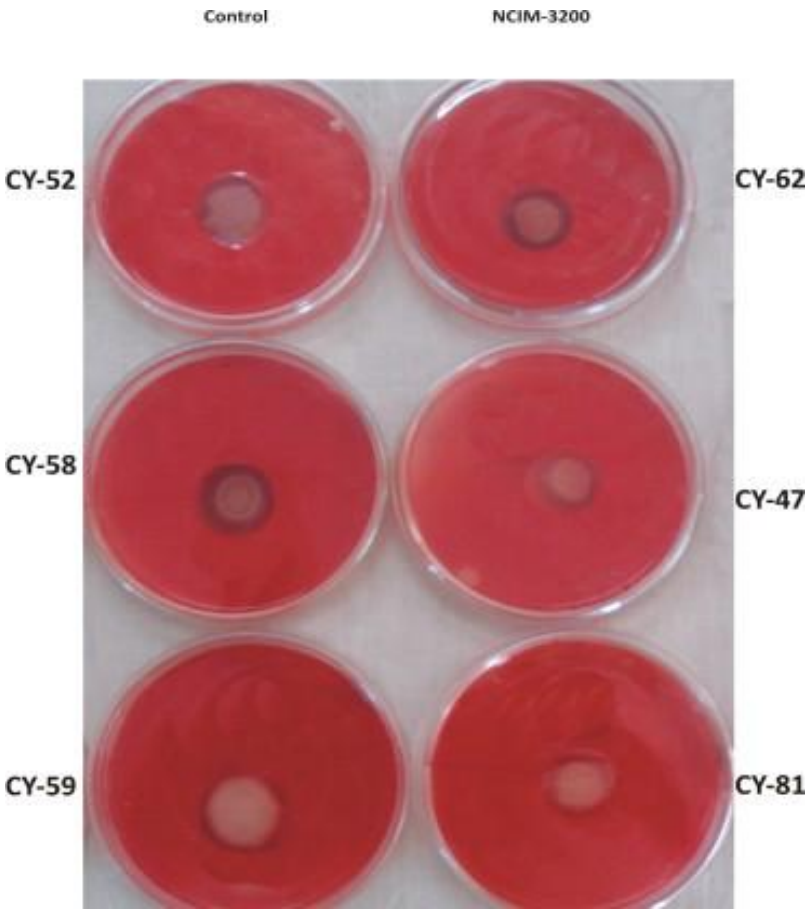


Plate 3: Yeast isolates showing zone of hydrolysis on CMC agar plate

Table 4: Release of reducing sugars (mg g^{-1}) by the yeast isolates at 5 days of interval

Sl. No	Isolates	Reducing sugars mg g^{-1} CMC			
		1 DAI	**5 DAI**	**10 DAI**	**15 DAI**
1	CY-1	0	54.98	87.96.	112.93
2	CY-2	0	16.33	29.54	65.02
3	CY-5	0	0	16	39
4	CY-7	0	24.65	59.09	106.98
5	CY-13	10.04	28.87	69.85	134.76
6	CY-14	0	20.64	30.76	64.34
7	CY-17	1.47	29.67	46.74	98.85
8	CY-18	0	0	17.33	59.76
9	CY-20	0	3.07	29.04	56.98
10	CY-24	0	3.04	39.54	89.03
11	CY-29	0	0.16	10.33	27.98
12	CY-30	5.03	29.44	78.09	105.98
13	CY-31	0	30.03	69.09	124.0
14	CY-32	0	42.09	88.09	128.08
15	CY-33	13.03	54.03	75.05	109.03
16	CY-34	0	3.05	39.04	69.04
17	CY-39	0	52.03	79.03	119.93
18	CY-40	2.04	16.94	28.08	65.03
19	CY-42	0	37.03	67.04	148.03
20	CY-43	0	34.02	48.02	89.04
21	CY-45	0	1.98	34.09	29.08
22	CY-47	8.02	24.06	34.08	67.03
23	CY-48	0.35	36.03	80.03	120.09
24	CY-52	**13.02**	**38.02**	**79.09**	**167.03**
25	CY-58	**10.03**	**29.03**	**74.03**	**159.83**
26	CY-59	**16.05**	**46.03**	**89.03**	**174.03**
27	CY-62	**14.03**	**43.03**	**83.03**	**170.03**
28	CY-65	0	0	23.02	34.02
29	CY-68	0	2.03	16.03	36.03
30	CY-81	**13.08**	**42.03**	**82.08**	**169.02**
31	CY-83	2.03	18.03	34.02	78.09
32	NCIM-3200	17.14	49.20	90.88	186.01
	S.Em±	0.29	0.77	0.87	1.14
	CD at1%	0.98	2.68	3.02	3.98

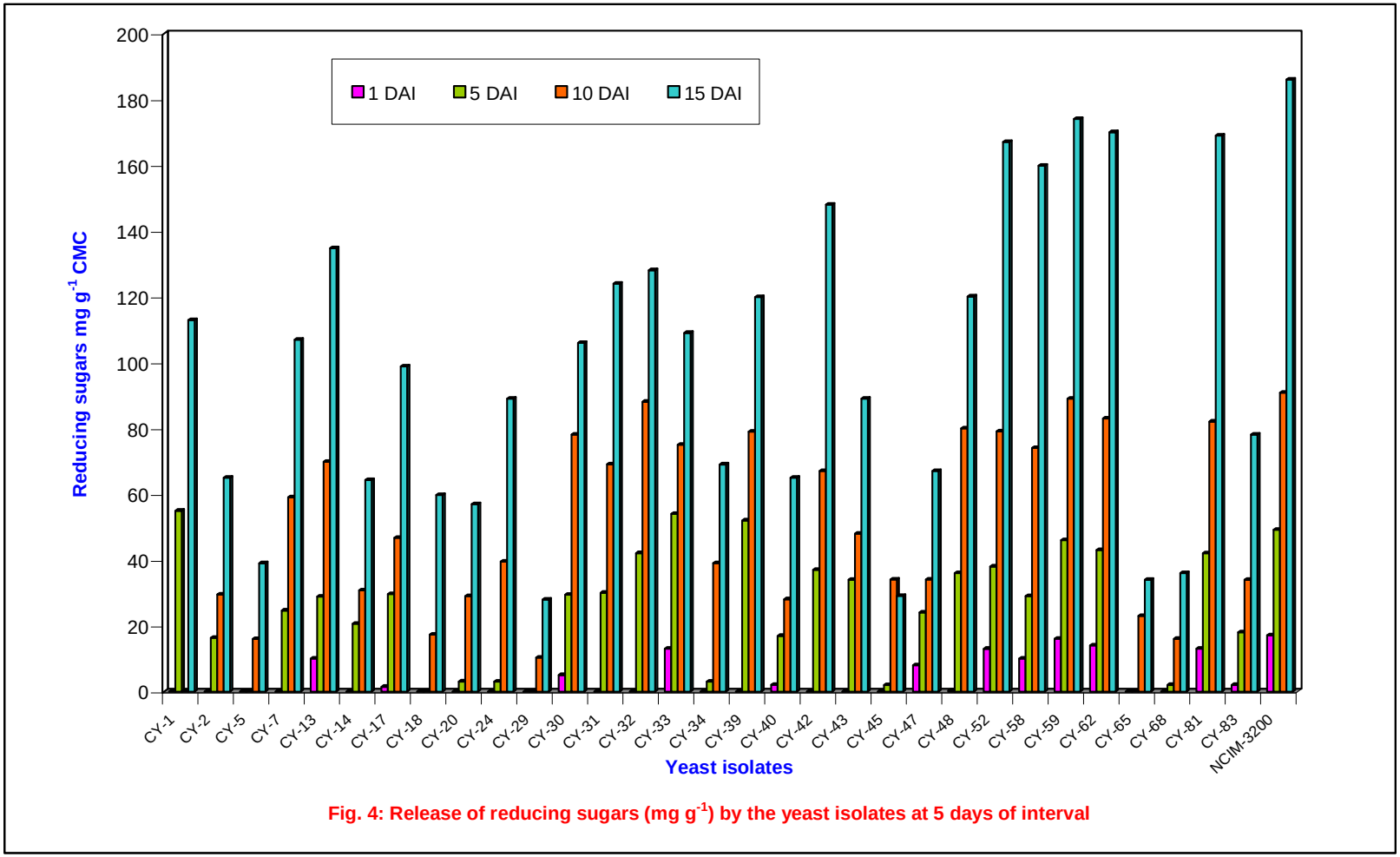


Fig. 4: Release of reducing sugars (mg g^{-1}) by the yeast isolates at 5 days of interval

Fig. 4: Release of reducing sugars (mg g-1) by the yeast isolates at 5 days of Interval

Table 5: Release of total sugars by the yeast isolates at 5 days of interval

Sl. No	Isolates	Total sugars mg g^{-1} CMC			
		1 DAI	**5 DAI**	**10DAI**	**15DAI**
1	CY-1	0	42.08	104.06	139.65
2	CY-2	0	22.05	34.09	78.05
3	CY-5	0	0	28	55
4	CY-7	0	35.07	70.43	116.98
5	CY-13	14.08	35.05	82.03	145.04
6	CY-14	0	27.04	39.03	80.87
7	CY-17	6.08	34.04	64.05	109.05
8	CY-18	0	0	23.07	70.94
9	CY- 20	0	7.09	34.06	69.09
10	CY- 24	0	7.07	45.98	106.09
11	CY- 29	0	3.97	17.08	40.98
12	CY- 30	13.98	40.05	89.09	123.06
13	CY- 31	0	43.08	83.02	139.07
14	CY- 32	0	52.08	102.08	165.08
15	CY- 33	19.08	39.05	104.06	132.09
16	CY- 34	0	7.08	45.06	84.06
17	CY- 39	0	44.08	93.05	138.05
18	CY- 40	5.08	29.06	43.08	88.08
19	CY- 42	0	36.08	84.08	172.09
20	CY- 43	0	39.05	73.06	112.08
21	CY- 45	0	5.08	53.08	56.09
22	CY- 47	14.08	32.08	53.08	81.09
23	CY- 48	3.08	42.09	96.09	145.09
24	CY- 52	**24.08**	**47.98**	**116.08**	**185.03**
25	CY- 58	**21.08**	**42.09**	**108.08**	**180.04**
26	CY- 59	**32.08**	**56.98**	**132.09**	**193.05**
27	CY- 62	**28.09**	**53.98**	**121.09**	**190.05**
28	CY- 65	0	0	38.09	65.08
29	CY- 68	0	5.07	34.08	56.08
30	CY- 81	**26.08**	**50.09**	**118.07**	**188.98**
31	CY- 83	9.05	38.08	68.09	104.08
	NCIM-3200	34.29	60.90	141.19	206.36
	S.Em ±	0.64	1.01	1.29	1.43
	CDat1%	2.06	3.09	3.94	4.02

DAI- Days after incubation

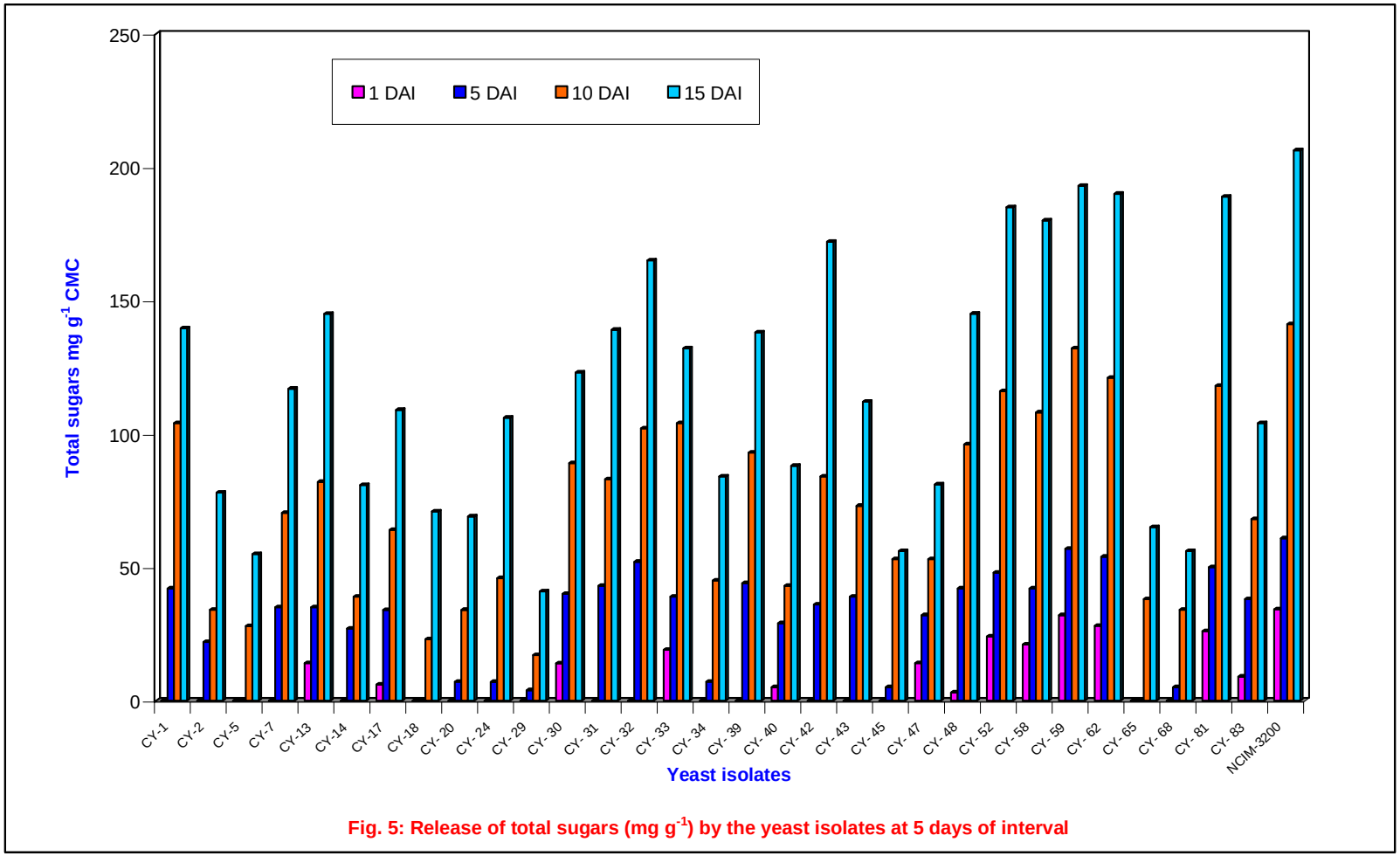


Fig. 5: Release of total sugars (mg g^{-1}) by the yeast isolates at 5 days of interval

Fig. 5: Release of total sugars (mg g-1) by the yeast isolates at 5 days of interval

Table 6: Release of sugars by the selected potential yeast isolates in CMC broth at 15 days of incubation

Sl. No	Isolates	Reducing sugars (mg g^{-1})	Non reducing residual sugars (mg g^{-1})	Total sugars (mg g^{-1})
1	CY-52	167.03	18.00	185.03
2	CY-58	159.83	20.21	180.04
3	CY-59	174.03	19.02	193.05
4	CY-62	170.03	20.02	190.05
5	CY-81	169.02	19.96	188.98
6	*S. cerevisiae* NCIM 3200	186.01	20.35	206.36

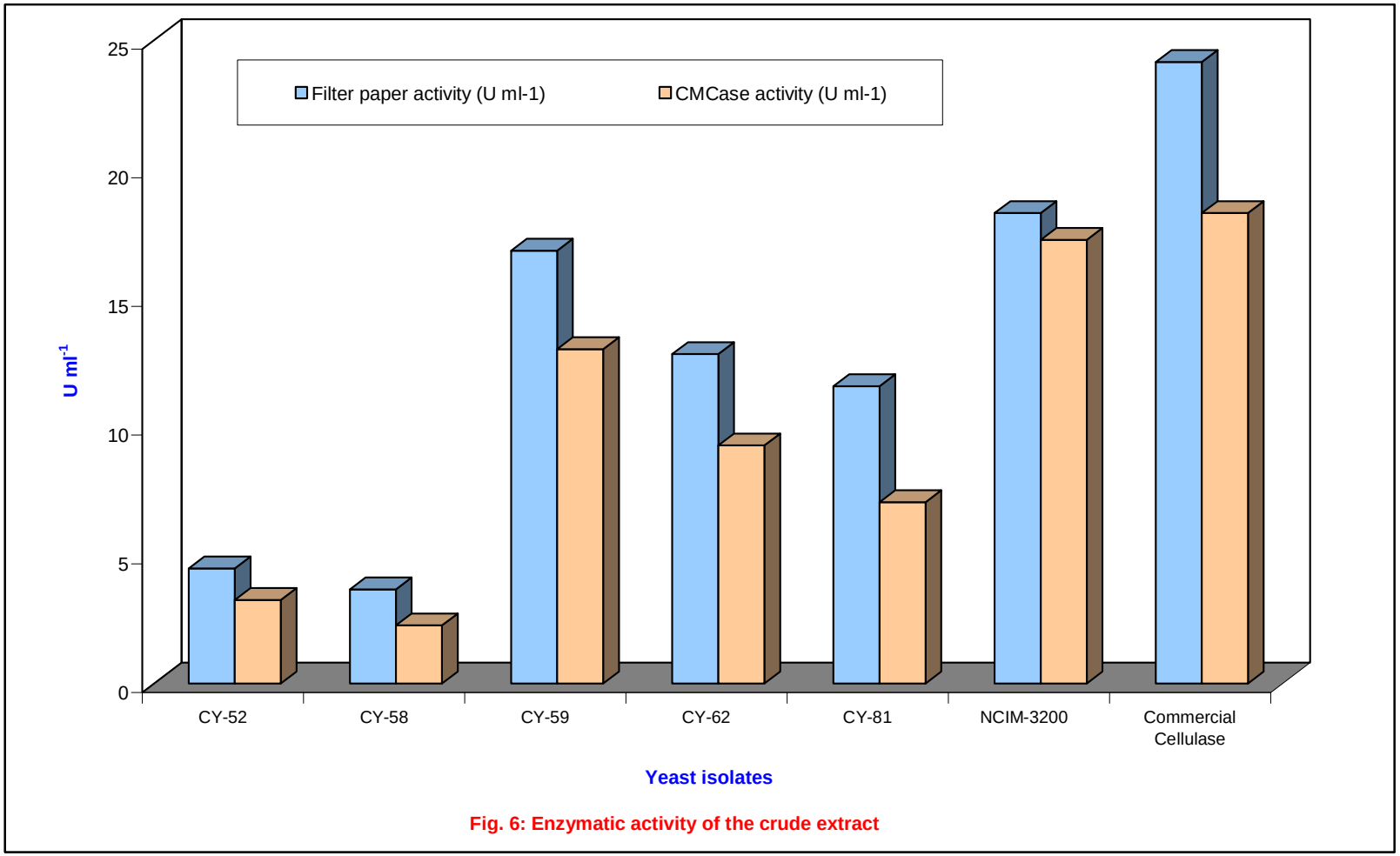


Fig. 6: Enzymatic activity of the crude extract

Fig. 6: Enzymatic activity of the crude extact

4.5 Screening of efficient isolates

Based on the zone of solubilization and release of reducing and total sugars, five strains were selected *viz:* CY-52, CY-58, CY- 59, CY- 62 and CY- 81. The *S. cerevisiae* NCIM 3200 strain was used as a reference strain.

4.6 Biochemical characterization of the isolates

Selected five isolates were tested for utilization of nine sugars viz: glucose, galactose, sucrose, maltose, lactose, starch, D-xylose, arabinose and D-ribose. All the strains utilized glucose, galactose, maltose and sucrose sugars where as except isolates CY- 58, all isolates unable to utilize lactose. Out of five isolates, CY-59 and CY-81 cannot utilize pentose sugars (Table 7).

4.7 Delignification of paddy straw and sugarcane baggase

Two substrates paddy straw and sugarcane baggase were taken whose initial cellulose content was estimated by Acid detergent fiber (ADF). The substrates were chemically pretreated to remove lignin. The cellulose thus obtained from the respective substrates was used for the further conversion.

4.7.1 Cellulose content in the substrates

Among the alkali (NaOH) pretreated substrates, sugarcane bagasse (5 mm particle size) has showed significantly higher cellulose content of 580 mg g^{-1} than paddy straw (489 mg g^{-1}). The 3 per cent alkali concentration has showed maximum cellulose content (577 mg g^{-1}) compared to 2.5 per cent alkali concentration (493 mg g^{-1}). The significant increase in cellulose content was recorded in 3 per cent NaOH pretreatment (695 mg g^{-1}) compared to 2.5 per cent NaOH (589 mg g^{-1}). The sugarcane bagasse has showed significant increase in cellulose content (695 mg g^{-1}), pretreated with 3 per cent alkali (NaOH) for 8 h of incubation period compared to all other substrates (Table 8).

Initial cellulose content was found to be 340 mg g^{-1}, 390 mg g^{-1} in paddy straw and sugarcane bagasse respectively.

4.8 Enzymatic assay to know the saccharification of the isolated strains

Enzyme assays such as filter paper activity, CMCase activity were performed to know enzymatic activity of the efficient strains along with the reference strain and commercial enzymes.

4.8.1 FPase activity

Filter paper activity was assayed to find out the efficiency of isolates activity which was expressed in terms of filter paper units (FPU). Among all isolates CY- 59 (16.82 U ml^{-1}) showed the highest activity followed by CY-62 (12.80 U ml^{-1}), CY-81 (11.55 U ml^{-1}), CY-58 (7.66 U ml^{-1}) and CY-52 (4.47 U ml^{-1}). Reference strain and commercial cellulase showed 18.28 U ml^{-1} and 24.15 U ml^{-1} respectively.

4.8.2 CMCase activity

Similarly CMCase activity was assayed for the isolates activity and was expressed in terms of U ml^{-1}. Among all the strains CY-59 (12.99 U ml^{-1}) showed the highest activity followed by CY-62 (9.25 U ml^{-1}), CY-81 (7.05 U ml^{-1}) CY-58 (3.26 U ml^{-1}) and CY-52 (2.25 U ml^{-1}). The CMCase activity of the reference strain and commercial cellulase was 17.24 U ml^{-1} and 18.28 U ml^{-1} respectively. (Table 9)

4.9 Performance of yeast isolates on reducing sugars release in Paddy straw and sugarcane bagasse

After delignification the selected isolates were allowed to grow on two substrates i.e. paddy straw and sugarcane bagasse. After 24 h initial concentration was measured in mg g^{-1}

The strain CY-59 (0.561 mg g^{-1}) showed the highest reducing sugars followed by CY-62 (0.196 mg g^{-1}),CY-81 (0.149 mg g^{-1}),CY-52 (0.122 mg g^{-1}) and CY-58 (0.055 mg g^{-1}) After 10 days final concentration was measured which was highest in case of CY-59 (2.7 mg g^{-1}) followed by CY-62 (1.627 mg g^{-1}), CY-81 (1.207 mg g^{-1}) CY-52 (0.451 mg g^{-1}) and CY-58 (0.378 mg g^{-1}).

Commercial cellulase showed the highest concentration of reducing sugars release and 15.967 mg g^{-1} than reference strain and 3.361 mg g^{-1} respectively. In similar way, uninoculated control showed final concentration of reducing sugar 0.015 mg g^{-1}.

Table 7: Utilization of sugars by the yeast isolates

Sl no	Isolates	Glucose	Maltose	Ribose	Lactose	Sucrose	Arabinose	Galactose	Xylose	starch
1	CY52	+	+	+	-	+	+	+	+	+
2	CY58	+	+	-	+	+	+	+	-	+
3	CY59	+	+	-	-	+	-	+	-	-
4	CY62	+	+	-	-	+	+	+	-	-
5	CY81	+	+	-	-	+	+	+	-	-
6	*S. cerevisiae* NCIM-3200	+	+	-	-	+	-	+	-	+

+ indicates growth

- indicates no growth

Table 8: Effect of alkali on paddy straw and bagasse at different period of incubation

Sl no	Substrate	Concentration of alkali(NaOH)	Cellulose content(mg g^{-1}) 6h	8h	Mean substrate
1	Paddy straw	2.5%	421	491	489
		3%	483	564	
2	Sugarcane bagasse	2.5%	471	589	580
		3%	567	695	
3	Mean concentration of alkali	2.5%	493		
		3%	577		
Source		S.Em±		CD at 1%	
Substrate (A)		0.0035		0.013	
Incubation period (B)		0.0029		0.011	
Concentration of alkali (C)		0.0029		0.011	
AXB		0.005		0.019	
AXC		0.005		0.019	
BXC		0.0041		NS	
AXBXC		0.007		NS	

Initial cellulose content
Paddy straw – 340 mg g^{-1}
Sugarcane bagasse – 390 mg g-^{1}

Table 9: Enzymatic activity of the crude extract

Sl. No.	Isolates	Reducing sugars	
		FPase activity ($U\ ml^{-1}$)	CMCase activity ($U\ ml^{-1}$)
1	CY-52	4.47	3.25
2	CY-58	3.66	2.26
3	CY-59	16.82	12.99
4	CY-62	12.80	9.25
5	CY-81	11.55	7.05
6	*Saccharomyces cerevisiae* NCIM-3200 (Reference strain)	18.28	17.24
7	Commercial Cellulase	24.15	18.28

Similarly in case of bagasse, reducing sugar was estimated after 15 days of incubation, which was found highest in case of CY-62 (2.880 mg g^{-1}) followed by CY-62 (1.980 mg g^{-1}), CY-81(1.227 mg g^{-1}), CY-52 (1.121 mg g^{-1}) and CY-58 (0.505 mg g^{-1}). This shows that there is significant variation of cell growth during the growth phase. Commercial cellulase and reference strain showed the highest concentration of 18.16 mg g^{-1} and 3.390 mg g^{-1} respectively. Similarly uninoculated control showed the reducing sugar of 0.079 mg g^{-1}. There is no change in the uninoculated control.

4.10 Estimation of ethanol from substrates

After 4 days of aerobic, anaerobic condition created for bioethanol production, ethanol was estimated after 5 days of fermentation. Among the all strains CY-59 showed the highest 48.18 mg g^{-1} followed by CY-62 (38.29 mg g^{-1}), CY-81(33.11 mg g^{-1}), CY-52 (28.11 mg g^{-1}) and CY-58 (21.99 mg g^{-1}) from paddy straw. Commercial cellulase and reference strain showed the highest value among all strain i.e. 72.29 mg g^{-1} and 64.62 mg g^{-1} respectively. Uninoculated control showed 3.8 mg g^{-1} bioethanol from paddy straw.

Similarly Strain CY-59 (60.51 mg g^{-1}) showed the highest value followed by CY-62 (49.51mg g^{-1}), CY-81 (43.88 mg g^{-1}), CY-52 (37.14 mg g^{-1}) and CY-58 (28.66 mg g^{-1}) from sugarcane bagasse. Commercial cellulase and reference strain showed the highest value of 76.99 mg $g^{-1}$and 72.44 mg g^{-1} respectively compared to all the isolates. Uninoculated control showed no change in the reducing sugars. (Table 10 and Plate 4).

The result indicates that, the yeast isolates were also efficiently exhibiting the cellulase activity and converting the sugars to alcohol, although less than that of commercial enzyme.

Table 10: Performance of yeast isolates in delignified paddy straw and bagasse in release of reducing sugars and bioethanol production

Sl. No	Strains	Paddy straw		Sugar cane bagasse	
		Reducing sugar (mg g^{-1})	Bioethanol (mg g^{-1})	Reducing sugar (mg g^{-1})	Bioethanol (mg g^{-1})
		15 days	5 days	15 days	5 days
1	CY-52	0.451	28.11	1.121	37.14
2	CY-58	0.378	21.99	0.505	28.66
3	CY-59	2.7	48.18	2.880	60.51
4	CY-62	1.627	38.29	1.980	49.51
5	CY-81	1.207	33.11	1.227	43.88
6	NCIM-3200	3.361	64.62	3.390	72.44
7	Commercial Cellulase	15.967	72.29	18.16	76.99
8	Uninoculated Control	0.015	3.8	0.079	3.8
	S.Em ± CD at 1%	0.148 0.52	1.51 5.25	0.174 0.61	2.07 7.25

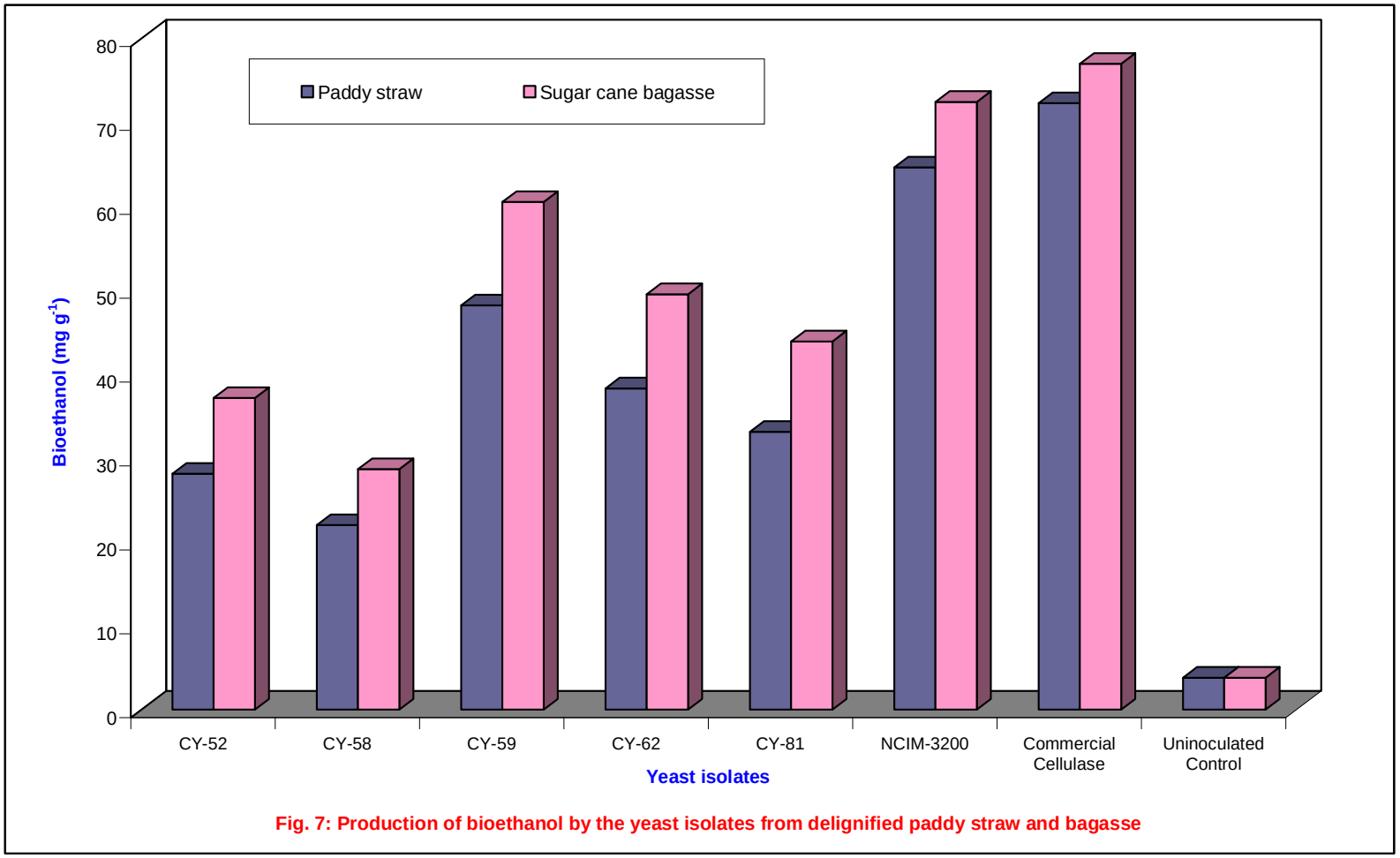


Fig. 7: Production of bioethanol by the yeast isolates from delignified paddy straw and bagasse

Fig. 7: Production of bioethanol by the yeast isolates from delignified paddy straw and bagasse

Plate 4: Pre-treated substrate used for bioethanol production

Plate 5: Experimental setup for fermentation of bioethanol

DISCUSSION

The recent increase in the oil price, shortage of fossil fuels and environmental pollution are the main reasons which are responsible for search of alternative energy sources in the recent days. In the past few years, the prices of petroleum products and the natural gas have been inflated. Therefore it is necessary to search for alternate energy sources, which are renewable, cost effective and environmentally safe. Petroleum industry now looks very committed to the use of the ethanol as fuel, as it is expected to benefit sugarcane growing farmers as well as the oil industry in the long run. Ethanol can be blended directly in petrol, up to 20 per cent. Presently, 5 per cent of ethanol is being blended along with diesel for transportation in few states in India. This indicates the demand for ethanol.

In India, ethanol is produced by fermentation of sugarcane molasses using strains of *S. cereviseae*. But the increase in the cost of molasses, it has been emphasized to search alternate substrates for ethanol production. Ethanol can also be produced from wheat, corn, beet, sweet sorghum *etc*. Apart from these, some of the alternate biomass tried successfully are the pineapple, cannery waste (Nigam, 1999), starch (Verma *et al*., 2000). Lignocellulosic biomass can be converted into fermentable sugars via various physicochemical and biological processing (Szczodrak and Fiedurek, 1996). The lignocellulosic biomass mainly includes wood waste, paper waste and crop residues like paddy straw, wheat straw, sugarcane bagasse *etc*. In India, paddy, wheat and sugarcane are the major cultivated crops and also in Karnataka. It is reported that 606 million tonne of paddy straw, 299 million tonne of wheat straw and 887 thousand tonnes of bagasse are being produced every year. These crop residues do have potential for production of ethanol as they contain considerable amount of sugars in the form of cellulose and hemicellulose.

In order to isolate the yeast sp., total seventeen samples were collected from different locations of North Karnataka from various natural sources *viz.* sugarcane field, forest litter, rotten fruits and compost. While collecting the samples Geographical Positioning System (GPS) was adopted to find out the particular latitude, longitude and elevation of the location. Based upon the budding character, slight yellowish, oval and round colonies were picked and selected for further study. A total of thirty one isolates were selected and screened on the basis of zone of hydrolysis. Among the thirty one isolates, five isolates were selected. Maximum zone of hydrolysis was observed in CY-59 followed by CY-62, CY-52, CY-81 and CY-58. Chatterjee *et al.,*(2011) isolated yeast strains was 12 from dahi, 2 each from apple juice, pineapple juice, mango juice, sweet lime juice, grape juice, orange juice, jaggery and 7 from sugarcane juice. These strains were found to produce various extra cellular enzymes and could ferment various carbon sources for the production of alcohol. Irfan *et al*. (2012), isolated several different bacterial strains from soil samples and screened for cellulase production in submerged fermentation process. Among these seven tested bacterial strains; ASN2 showed maximum yield for cellulase production. This strain was further characterized by biochemical and morphological tests and identified as *Cellulomonas* sp. ASN2.

Thirty one isolates were screened in CMC broth to know the efficiency of the isolates in terms of releasing reducing sugars. There is considerable variation in the initial degradation of CMC among the strains. The isolates CY-5, CY-18 and CY-65 showed delayed response towards CMC broth in releasing reducing sugars indicating absence of all three cellulase enzymes in different incubation days. Out of 31 yeast isolates, 13 isolates viz: CY-13, CY-17, CY-30, CY-33, CY-40, CY-47, CY-48, CY-52, CY-58, CY-59, CY-62, CY-65 CY-68, CY-81 and CY-83 showed the immediate response towards degradation of cellulose indicating possibility of presence of all three cellulase enzymes in different incubation days.

Among all isolates screened for reducing sugars, the isolate CY-59 showed the highest reducing sugars followed by CY-62, CY-81, CY 52 and CY-58. The viscosity of the CMC broth was reduced. Lignocellulosics contain polysaccharides like cellulose (50%), hemicellulose (25%) and lignin (25%) (Ashok *et al*., 2000). These polysaccharides after pretreatment break down into simple sugars, which is subsequently fermented to ethanol by fermenting microorganisms. Initially to delignify the substrates, different physical and chemical pretreatments methods were employed.

Out of 31 isolates, 13 isolates CY-13, CY-17, CT-30, CY-33, CY-40, CY-47, CY-48, CY-52, CY-58, CY-59, CY-62, CY-65 CY-68, CY-81 and CY-83 showed the immediate response towards degradation of cellulose indicating possibility of presence of all three cellulase enzymes in different incubation days.

Among all isolates CY-59 showed the highest total sugars followed by CY-62, CY-81, CY-52 and CY-58. Further non reducing residual sugars were calibrated in strains CY-52, CY-58, CY-59, CY-62 and CY-81.

Based on the zone of solubilization and concentration of reducing and total sugars, five strains were selected *viz:* CY-52, CY-58, CY- 59, CY- 62 and CY- 81. The *S. cerevisiae* NCIM 3200 strain was used as a reference strain. All the isolates utilized glucose, galactose and maltose and sucrose sugars where as except strain CY- 58, all isolates unable to utilize lactose. Out of five isolates, CY-59 and CY-81 cannot utilize pentose sugar. Qureshi *et al*. (2007), isolated yeast strains from control (saf instant), citrus juice, dahi and sugarcane juice was 1, 14, 13 and 12, respectively. From these 40 strains, 14 were identified as *S. cerevisiae*, 12 as *S. kluyveri*, 4 as *S. exigus* and *S. dairnensis*, 2 as *S. ludwigii, S. octosporus* and *S. unisporus,* respectively. Later on, 14 isolates of *S. cerevisiae* were assessed for their maltose utilization capacity for bread making.

Two substrates paddy straw and sugarcane bagasse were selected whose cellulose content was estimated by Acid detergent fiber (ADF).

Among the alkali (NaOH) pretreated substrates, sugarcane bagasse (5 mm particle size) showed significantly higher cellulose content of 580 mg g^{-1} than paddy straw (489 mg g^{-1}). The 3 per cent alkali concentration showed maximum cellulose content (577 mg g^{-1}) compared to 2.5 per cent alkali concentration (493 mg g^{-1}). The significant increase in cellulose content was recorded in 3 per cent NaOH pretreatment (695 mg g^{-1}) compared to 2.5 per cent NaOH (589 mg g^{-1}). The sugarcane bagasse showed significant increase in cellulose content (695 mg g^{-1}), pretreated with 3 per cent alkali (NaOH) for 8 h of incubation period compared to all other substrates. Initial cellulose content was found to be 340 mg g^{-1}, 390 mg g^{-1} in paddy straw and sugarcane bagasse respectively. Similar work was done by Shankarappa (2013) reported that treatment combination of 3% NaOH coupled with 121^0 C temp with particle size 0.5 mm was found to be best for the recovery of both cellulose and hemicellulose after pretreatment sugarcane bagasse (0.813 g g^{-1}), Sugarcane top (0.786 g g^{-1}), Sugarcane trash (0.806 g g^{-1}) corn husk (0.806 g g-1) and corn stover (0.806 g g^{-1}).

The difference in chemical composition of the agro-residues is due to variation in the composition of cell walls as it varies with plant species, tissue type and, region within the cell wall and development stages of the cell wall (Carpita and Mc Cann, 2000). Analysis of the substrates indicates that sugarcane bagasse and sugarcane trash contains high amounts of cellulose and could be potential substrates for ethanol production. The substrates were tested at different concentration of alkali in order to assess the extent of delignification and hydrolysis in releasing maximum reducing sugars.

Filter paper activity was assayed to know the efficiency of isolates activity which was expressed in terms of filter paper units (FPU). Among all strains CY- 59 showed the highest activity followed by CY-62, CY-81, CY-58 and CY-52. Reference strain and commercial cellulase showed 18.28 U ml^{-1} and 24.15 U ml^{-1} respectively. Sangkharak *et al.* (2011) showed *Cellulomonas sp*. strain TSU-03 produced the highest activity of xylanase and endoglucanase at 1860.1 and 388.5 U mg $^{-1}$ protein respectively.

Similarly CMCase activity was assayed for the isolates activity and result was expressed in terms of U ml^{-1}. Among all the strains CY-59 showed the highest activity followed by CY-62, CY-81 CY-58 and CY-52. The CMCase activity of the reference strain and commercial cellulase was 17.24 U ml^{-1} and 18.28 U ml^{-1} respectively. Similar work was done by Samira *et. al*. (2011) to know cellulase activity for *S. variabilis, K. rosea, S. maltophilia* and value was obtained after 24 hr of fermentation with 0.091, 0.089, 0.084 U ml^{-1} for CMCase and 0.079, 0.074 and 0.072 Uml^{-1} for FPase activity respectively. It clearly shows that there is release of three enzymes to degrade the cellulose.

After delignification the selected isolates were subjected to two substrates i.e. paddy straw and sugarcane bagasse. After 24 h initial concentration was measured in mg g^{-1} .The strain CY-59 showed the highest reducing sugars followed by CY-62, CY-81, CY-52 and CY-58. After 15 days final concentration was measured which was highest in case of CY-59 followed by CY-62, CY-81 CY-52 and CY-58 . Irfan *et al*, 2011 reported that100 g of sugar cane bagasse and wheat straw were treated with various concentrations of H_2O_2 (1-5% v/v) and its combination with 2% NaOH for one hour under steam at 30 psi. Sugarcane bagasse was 88% delignified with 5% H_2O_2 + 2% NaOH whereas wheat straw showed 51% delignification with 3% H_2O_2 + 2% NaOH. Moreover the saccharification with commercial cellulase enzyme was found to be 33.6% and 63.3% with pretreated wheat straw and sugarcane bagasse, respectively.

Commercial cellulase showed the highest concentration of final reducing sugar i.e. 15.967 mg g^{-1} than reference strain concentration 3.361 mg g^{-1}. Similarly uninoculated control showed the final concentration of reducing sugar 0.148 mg g^{-1}.

After 10 days of incubation, final concentration was estimated which was found highest in case of CY-59 (2.880 mg g^{-1}) followed by CY-62 (1.980 mg g^{-1}), CY-81(1.227 mg g^{-1}), CY-52(1.121 mg g^{-1}) and CY-58(0.505 mg g^{-1}). This shows there is significant variation of cell growth during the growth phase. Similar work has done by Gurav and Geeta, (2007) who reported that bagasse recorded maximum release of ethanol compared to paddy straw and wheat straw. They showed maximum production of ethanol 820.80 mg L^{-1} and *Zymomonas mobilis* was efficient in conversion to ethanol from all the substrates.

Commercial cellulose and reference strain showed the highest concentration of 18.16 mg g^{-1} and 3.390 mg g^{-1} respectively. Similarly uninoculated control showed the initial and final concentration of 0.079 mg g^{-1} and 0.079 mg g^{-1} respectively. It showed there is no change in uninoculated control.

After 4 days of aerobic followed by anaerobic fermentation bioethanol was estimated. Among the all strains CY-59 showed the highest 48.18 mg g^{-1} followed by CY-62 (38.29 mg g^{-1}), CY-81(33.11 mg g^{-1}), CY-52 (28.11 mg g^{-1}) and CY-58 (21.99 mg g^{-1}) from paddy straw. Commercial cellulase and reference strain showed the highest value among all strain i.e. 72.29 mg g^{-1} and 64.62 mg g^{-1} respectively. Uninoculated control showed 3.8 mg g^{-1} from paddy straw that showed naturally decomposed material has some significance of bioethanol production. Massoutier *et al* (1998) reported that eleven cryotolerant strains produced twice as many isobutyl and isoamyl alcohols as mesophilic yeast and 2-phenethyl alcohol was produced by cryotolerant yeasts at levels 4 times as high as by mesophilic yeast.

Similarly strain CY-59 showed the highest bioethanol production followed by CY-62, CY-81, CY-52 and CY-58 from sugarcane bagasse. Commercial cellulase and the reference strain showed the highest value of 76.99 mg g^{-1} and 72.44 mg g^{-1} respectively among all the isolates. Uninoculated control showed 7.25 mg g^{-1} from bagasse. Similar comparative study of ethanol production was carried out by Patel *et al* (2012). Rice husk and bagasse hydrolyzates obtained after fungal treatment were fermented using different yeasts like *Saccharomyces cerevisiae*-NCIM 3095*, S. uvrum* –NCIM 3455, *Schizosaccharomyces cerevisiae pombe*-NCIM-3457, *Candida shehatae*-NCIM-3500, *Pichia stipitis*-NCIM-3498. Separate hydrolysis and fermentation method was found to give better result of ethanol.

Thus, it is evident that the yeast can exhibit the cellulase activity when cellulose substrate is available and can convert to alcohol also although the yields are less than the commercial cellulase enzyme. The strain CY-59 has shown maximum saccharification as well as alcohol production. It was well established that the strain exhibited all three components of cellulase enzyme. However, extrapolating more sources from nature can yield better strains. The reference strains also need to be studied in detail for expression of the cellulolytic activity.

SUMMARY AND CONCLUSION

In order to isolate the yeast sp. total one hundred four isolates were collected from different locations of North Karnataka from various natural sources *viz.* sugarcane field, forest litter, rotten fruits and compost. While collecting the samples, Geographical Positioning System (GPS) was adopted to find out the particular latitude, longitude and elevation of the location. All the isolates were tested for zone of hydrolysis in carboxy methyl cellulose media and maximum zone of hydrolysis was observed in CY-59 (29 mm) followed by CY-62 (28 mm), CY-52 (27 mm), CY-81 (26 mm) and CY-58 (24 mm).

In order to know the efficiency of the isolates in terms of releasing reducing sugars, all the isolates were screened in CMC broth. Among all isolates screened for reducing sugars the CY-59 showed the highest reducing sugars (174.03 mg g^{-1}) followed by CY-62 (170.03 mg g^{-1}), CY-81 (169.02mg g^{-1}), CY 52 (167.03 mg g^{-1}) and CY-58 (159.83 mg g^{-1}) and CY-13, CY-17, CY-30, CY-33, CY-40, CY-47, CY-48, CY-52, CY-58, CY-59, CY-62, CY-65 CY-68, CY-81 and CY-83 showed the immediate response towards degradation of cellulose indicating possibility of presence of all three cellulase enzymes in different incubation days.

Among all the isolates CY-59 showed the highest total sugars (193.05 mg g^{-1}) followed by CY-62 (190.05 mg g^{-1}), CY-81 (188.98 mg g^{-1}),CY-52 (185.03 mg g^{-1}) and CY-58 (180.04 mg g^{-1}). The non reducing residual sugars were CY-52 (18 mg g^{-1}), CY-58 (20.21 mg g^{-1}), CY-59 (19.02 mg g^{-1}), CY-62 (20.02 mg g^{-1}) and CY-81 (19.96 mg g^{-1}).

Based on the zone of solubilization and the concentration of reducing and total sugars, five strains *viz.,* CY-52, CY-58, CY- 59, CY- 62 and CY- 81 were found efficient and selected for substrate analysis and *S. cerevisiae* NCIM 3200 was used as a reference strain. Two substrates paddy straw and sugarcane bagasse were selected and cellulose content was estimated by Acid detergent fiber (ADF).

Among the alkali (NaOH) pretreated substrates, sugarcane bagasse (5 mm particle size) showed significantly higher cellulose content of 580 mg g^{-1} than paddy straw (489 mg g^{-1}). The 3 per cent alkali concentration showed maximum cellulose content (577 mg g^{-1}) compared to 2.5 per cent alkali concentration (493 mg g^{-1}). The significant increase in cellulose content was recorded in 3 per cent NaOH pretreatment (695 mg g^{-1}) compared to 2.5 per cent NaOH (589 mg g^{-1}). The sugarcane bagasse showed significant increase in cellulose content (695 mg g^{-1}), pretreated with 3 per cent alkali (NaOH). Initial cellulose content was found to be 340 mg g^{-1}, 390 mg g^{-1} in paddy straw and sugarcane bagasse respectively. Enzyme assays such as filter paper activity, CMCase activity were performed to know the enzymatic activity of the efficient strains along with the reference strain and commercial enzymes were used for bio-ethanol production.

Among all strains CY- 59 (16.82 U ml^{-1}) showed the highest FPase activity followed by CY-62 (12.80 U ml^{-1}), CY-81 (11.55 U ml^{-1}), CY-58 (7.66 U ml^{-1}) and CY-52 (4.47 U ml^{-1}). Reference strain and commercial cellulase showed 18.28 ml^{-1} and 24.15 U ml^{-1} respectively.

Similarly, CMCase activity was assayed to know the enzyme activity of the isolates, out of all the strains CY-59 (12.99 U ml^{-1}) showed the highest activity followed by CY-62 (9.25 U ml^{-1}), CY-81 (7.05 U ml^{-1}) CY-58 (3.26 U ml^{-1}) and CY-52 (2.25 U ml^{-1}). The CMCase activity of the reference strain and commercial cellulase was 17.24 U ml^{-1} and 18.28 U ml^{-1} respectively.

After delignification, the selected isolates were subjected to two substrates i.e. paddy straw and sugarcane bagasse. The strain CY-59 (0.561 mg g^{-1}) showed the highest reducing sugars followed by CY-62 (0.196 mg g^{-1}), CY-81 (0.149 mg g^{-1}), CY-52 (0.122 mg g^{-1}) and CY-58 (0.055 mg g^{-1}) in paddy straw and after 10 days of fermentation, the final concentration was highest in of CY-59 (2.7mg g^{-1}) followed by CY-62 (1.627 mg g^{-1}), CY-81 (1.207 mg g^{-1}) CY-52 (0.451 mg g^{-1}) and CY-58 (0.378mg g^{-1}). Commercial cellulase showed the highest concentration of reducing sugar in initial and final i.e. 3.024 mg g^{-1} and 15.967 mg g^{-1} than the reference *S. cerevisiae* NCIM 3200 1.831 mg g^{-1} and 3.361 mg g^{-1} respectively.

Similarly in case of bagasse initial concentration was found highest in case of CY-59 (1.095 mg g^{-1}) followed by CY-62 (0.753 mg g^{-1}), CY-81 (0.726 mg g-$^{-1}$), CY-52 (0.568 mg g^{-1}) and CY-58 (0.128 mg g^{-1}). Commercial cellulase and reference strain showed the highest concentration of 3.645 mg g-$^{-1}$ and 2.494 mg g^{-1} respectively. After 10 days of fermentation, final concentration was estimated which was found highest in case of CY-62 (2.880 mg g^{-1}), followed by CY-62 (1.980 mg g^{-1}), CY-81 (1.227 mg g^{-1}), CY-52 (1.121 mg g^{-1}) and CY-58 (0.505 mg g^{-1}). This shows there is significant variation of cell growth during the growth phase.

Among the all strains CY-59 showed the highest 48.18 mg g^{-1} bioethanol production followed by CY-62 (38.29 mg g^{-1}), CY-81 (33.11 mg g^{-1}), CY-52 (28.11 mg g^{-1}) and CY-58 (21.99 mg g^{-1}) from paddy straw. Commercial cellulase and reference strain showed the highest value among all strain i.e. 72.29 mg g^{-1} and 64.62 mg g^{-1} respectively.

Similarly Strain CY-59 (60.51 mg g^{-1}) showed the highest value followed by CY-62 (49.51mg g^{-1}), CY-81 (43.88 mg g^{-1}), CY-52 (37.14 mg g^{-1}) and CY-58 (28.66 mg g^{-1}) from sugarcane bagasse. Commercial cellulase and reference strain showed the highest value of 76.99 mg $g^{-1}$and 72.44 mg g^{-1} respectively among all the isolates.

Thus among the five efficient strains, strain- 59 showed the highest reducing sugar and bioethanol production, which is somewhat competitive to *S. cerevisiae* NCIM - 3200 and commercial cellulase. The isolated cellulolytic yeast can be further constructed through genetic engineering for more efficiency for bioethanol production.

The yeast strains exhibited cellulase activity when cellulose substrate was provided and can convert to alcohol also although the yields are less than the commercial cellulase enzyme. The strain CY-59 has shown maximum saccharification as well as alcohol production. It was well established that the strain exhibited all the three components of cellulase enzyme. However, extrapolating more sources from nature can yield better strains. The reference strains also need to be studied in detail for expression of the cellulolytic activity.

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Annexure I: Media preparation

Malt Extract Glucose Yeast Extract Peptone (NCIM Catalogue, India, 2002)

Sl. No.	Name of Component	(g L^{-1})
1	Yeast Extract	3.0
2	Malt Extract	3.0
3	Glucose	10.0
4	Peptone	5.0
5	Agar	18.0
	Distilled water	1000 ml
6	pH	6.4 – 6.8
7	Streptomycin	0.25 mg/100 ml

Carboxy methyl cellulose (CMC) agar medium

Sl. No.	Name of Component	(g L^{-1})
1	$NaNO_3$	3.0
2	K_2HPO_4	3.0
3	KCl	10.0
4	$MgSO_4$	5.0
5	Yeast Extract	0.5
6	Glucose	18.0
7	Carboxy methyl Cellulose	5.0
8	Agar	18.0
9	pH	6.4 – 6.8
10	Distilled water	1000 ml

ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC YEASTS FOR BIOETHANOL PRODUCTION

S. C. SAHOO **2013** **Dr. GEETA G. SHIRNALLI**
Major advisor

ABSTRACT

The present study was conducted in the Department of Agricultural Microbiology on bioethanol production from selected agro-residues with the objectives whether two step process i.e. hydrolysis and fermentation could be transformed into single step with the help of efficient cellulolytic yeast strains, thus reducing the cost of bioethanol production.

A total of 104 yeast isolates from various natural habitat mainly rotten fruit rinds, forest leaf litter, compost, fruit samples, food stuff and over matured paragrass were isolated on the basis of zone of hydrolysis of cellulose and release of reducing sugars. Among these 31 cellulolytic yeasts were screened. Five efficient strains CY-52, CY-58, CY-59, CY-62 and CY-81 were chosen on the basis of release of maximum reducing sugars. The isolates were subjected for utilization of various carbon sources namely glucose, maltose, ribose, lactose, sucrose, arabinose, galactose, xylose and starch to know the sugar utilization efficiency.

The efficient yeast strains were subjected on two delignified substrates i.e. paddy straw and sugarcane bagasse. Delignification was carried out using alkali at 3 per cent for 8 h. Recovery of cellulose was highest in case of sugarcane bagasse i.e. 695 mg g^{-1} as compared to paddy straw 564 mg g^{-1}.To know the enzymatic assay, filter paper activity and CMCase activity was conducted with the efficient strains out of which strain CY-59 isolate showed the highest activity i.e. 16.82 U ml^{-1} and 12.99 U ml^{-1} respectively.

The yeast isolate CY-59 showed the maximum bioethanol production of 48.18 mg g^{-1} and 60.51 mg g^{-1} from paddy straw and sugarcane bagasse respectively which was on par to reference strain NCIM-3200and commercial cellulase. Thus, it can be concluded that yeasts are able to induce cellulase for hydrolysis of the substrates and produce ethanol. Extrapolating more sources from nature can yield better strains.