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INTEGRATED PROCESS FOR BIOCONVERSION OF PADDY STRAW, CHEESE WHEY AND MOLASSES INTO ETHANOL

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ABSTRACT

The present study on co-fermentation of paddy straw and cheese whey was carried out to assess the efficacy of whey as nutritional supplement for bio-ethanol production. Also, co-fermentation of paddy straw and molasses was carried out for enhanced ethanol production to make downstream processing and purification process easier. For agro-residues like paddy straw, breaking of recalcitrant lignin sheath is the first and success determining step in bioethanol production. To achieve this efficiently, five factors optimized were temperature, pressure, pH, residence time and particle size of paddy straw. Paddy straw (particle size: 0.5 mm) immersed in 2% sodium hydroxide solution (pH 10.0) in 1:10 was subjected to delignification using steam collected under pressure (temperature 121°C; pressure 15 psi) for 1 h. This resulted in 83.3% lignin removal with 86.4% cellulose and 11.1% hemicellulose recovery. Polysaccharide rich substrate was saccharified using commercial enzyme loaded at 7.5 FPU g^{-1} substrate at 50°C for 2 h. Hydrolyzed substrate was fermented with yeast strain *Candida utilis*. The combined fermentation of paddy straw with cheese whey increased ethanol production from 29.0 mL L^{-1} to 31.0 mL L^{-1} without yeast nutrient supplementation and with molasses increased to 51.0 mL L^{-1} ethanol with yeast nutrient supplementation.

Keywords: Bioethanol, C*andida utilis.* cheese whey, molasses, paddy straw

INTRODUCTION

The complex socio-economic and environmental issues related to energy use have forced the scientific communities to search reliable, renewable and economically viable alternatives. The economy of a country is greatly influenced by the energy cost of the country. Though fossil fuel is the dominant energy source worldwide but its limited availability and excessive use has posed serious global problem of energy resource scarcity. The renewable energy sources, including bioethanol, have gained huge attention across the world. Indigenous, non-polluting and virtually inexhaustible renewable resources are the most promising energy source in present times. Sugar and starch based raw materials like sugarcane, corn, grain wheat etc. are the part of human and animal food chain so their diversion for bioethanol production can lead to food shortage and price rises (Goel and Wati 2013a). Lignocellulosic biomass, not competing with the food chain, can provide environmental, economic and strategic benefits for the production of bioethanol (Viikari *et al*., 2012) and is considered as green alternative to fossil fuel (Toor *et al.*, 2020). Feasibility of various lignocellulosic materials for ethanol production has been studied all over the world depending upon their availability (Yadav *et al*., 2011; Goel and Wati, 2013b; Beniwal *et al*., 2018). Rice is the major crop grown worldwide and marked 500 million metric tonnes production globally in 2020-2021 as per estimate of USDA [*www.worldagriculturalproduction.com/crops/rice.aspx*].

The straw, farm waste, is traditionally burned in open field which pollutes air quality and leads to pulmonary morbidity and mortality (Goel and Wati, 2016). The carbohydrate in straw can serve as a feed stock for bioethanol. In present study we explored bioconversion of paddy straw with byproduct of two industries *viz*., cheese whey of dairy industry and molasses of sugar industry. Cheese whey is generally considered as waste and is cumbersome to handle due to its production in large amount and high biological- and chemical-oxygen demand (BOD and COD). However, it can serve as nutritional supplement for yeast in lignocellulosic bioethanol production (Zohri *et al.,* 2014). Supplementation of molasses to paddy straw hydrolysate can significantly enhance the overall ethanol yield making the downstream processing and purification of ethanol feasible for large scale industrial production. Although fermentation of composite sugars by simultaneous saccharification and co-fermentation of agro-residues like sugarcane bagasse (Gubicza *et al.,* 2016), rice straw (Goel and Wati, 2016), corn stover (Liu *et al.,* 2019) and sweet sorghum (Zhang *et al.*, 2019) has been reported by many researchers but combined fermentation of two different industrial wastes may give new insight for better waste management.

MATERIALS AND METHODS

Paddy straw variety ‘Pusa-1’ was procured from farmer’s field village Dabra, Hisar, Haryana (India). Cheese whey was obtained from local dairy and molasses from sugar mill Rohtak, Haryana, India. A commercial preparation of cellulase enzyme (Palkosoft super 720) was supplied by Maps India Ltd. Ahmedabad, Gujarat (India). The yeast strain *Candida utilis* was procured from the Department of Microbiology, CCS HAU, Hisar (India) and maintained on yeast extract peptone dextrose agar slants containing dextrose 20.0; yeast extract 10.0; peptone 20.0 and agar-agar 20.0 (g L^{-1}) by regular sub-culturing and stored at 4±1°C till further use.

Alkali pretreatment

Dried (moisture-free) paddy straw was reduced to particle size of 0.5, 1.0, 2.0 and 4.0 mm by using Wiley grinder fitted with sieves of different mesh size. Delignification of straw was done by alkali treatment (2% sodium hydroxide) [1:10 solid: liquid] at 121°C at 15 psi for 1 h in an autoclave (Wati *et al*., 2007). After cooling, straw was washed to get rid of alkali, neutralized with 0.1 M acetic acid and dried in hot air oven at 50°C.

Saccharification

The alkali treated paddy straw was immersed in citrate buffer at 1:10 (solid: liquid). The enzyme was loaded at 7.5 FPU g^{-1} substrate and hydrolysis carried out in shaking water bath at 50˚C. Total reducing sugars released were measured by standard DNS method (Miller, 1959) after centrifuging the samples at 5,000 rpm for 10 min. In Co-fermentation of paddy straw and cheese whey, cheese whey was added at 5.0% to the slurry of alkali treated paddy straw in citrate buffer.

Fermentation

The hydrolysate obtained after saccharification was fermented using *C. utilis*. The yeast inoculum was raised in YEPS medium containing yeast extract 5.0, peptone 5.0 and sucrose 60.0 (g L^{-1}) at 30°C. Yeast biomass obtained after 24h of incubation was centrifuged at 5,000 rpm for 15 min and inoculated into the hydrolysate at a concentration of 1.0% (w/v). The ethanol production potential of yeast strain was studied in co-fermentation of paddy straw hydrolysate with cheese whey and molasses at 35°C under stationary conditions. The effect of yeast nutrient *i.e.* 0.3% urea addition was investigated on the fermentation potential of yeast strain. The ethanol produced in fermented broth was estimated after 72 h as per the method of Caputi *et al*. (1968).

Analytical methods

Paddy straw was analyzed for cellulose, hemicellulose, lignin, acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents as per the standard methods (AOAC, 1970). The total organic carbon and ash contents were estimated as per volatile solids valuation method (AOAC, 1970) while total nitrogen and protein content was estimated by standard Kjeldahl's method (AOAC, 1970). Cheese whey was analyzed for total sugars as per method of Yemm and Willis (1954). Proteins, nitrogen and phosphorus were estimated as per the methods of Lowry *et al.* (1951), AOAC (1970) and John (1970), respectively. Lignin was extracted from pre-treatment solution by the method of Tian *et al.* (2015).

Statistical analysis

All the experiments were carried out in a completely randomized design with each treatment replicated three times. The data was statistically by using OPSTAT software available on CCSHAU home page. To compare the treatment, critical difference (P = 0.05) was calculated (Sheoran *et al*., 1998).

RESULTS AND DISCUSSION

Alkali pre-treatment

Paddy straw contained 35.1% cellulose, 24.8% hemicellulose, 6.3% lignin, 1.2% total nitrogen, 11.7% crude protein, 16.0% ash and 49.82% total organic carbon (Table 1). Cheese whey with total solids of 4.84% was acidic in nature (pH, 5.6). Total nitrogen, phosphorus and sugar contents of cheese were 0.70, 1.13 and 2.88%, respectively (Table 1). Polysaccharide fraction of paddy straw indicated that it contains a reasonable amount of sugars for fermentation to ethanol. The nitrogen and phosphorus content of cheese whey showed its potential to serve as nutritional supplement for yeast in fermentation process. The composition of paddy straw varies with variety, climatic conditions and other factors but it may be fairly compared with the findings of Yadav *et al.* (2011) who reported 35% cellulose, 22% hemicellulose and 12% lignin in paddy straw.

The lignin content (6.29%) of paddy straw showed that some pre-treatment is required to break this protective sheath and make cellulose and hemicellulose amenable to hydrolysis. In present study, alkali treatment was adopted due to its advantages like recovery of hemicellulose in solid fraction and formation of lesser fermentation inhibitors (furfurals). Particle size of paddy straw influences the delignification efficiency. Larger the available surface area better the penetration of alkali for lignin disruption. Treatment of variable mesh sized paddy straw (0.5, 1.0, 1.5, 2.0 and 4.0 mm) with 2%

Table 1: Proximate analysis of paddy straw and cheese whey

Paddy straw		Cheese whey	
Composition	Content (%w/v)	Composition	Content (%w/v)
Cellulose	35.07 ± 0.42	Total phosphorus	1.13 ± 0.08
Hemicellulose	24.85 ± 0.37	Soluble protein	0.39 ± 0.04
Lignin	6.29 ± 0.19	Total solids	4.84 ± 0.01
Total nitrogen	1.24 ± 0.06	Total sugars	2.88 ± 0.04
Crude protein	11.7 ± 0.24	Total nitrogen	0.70 ± 0.09
Ash	16.0 ± 0.26	pH	5.6
Organic carbon	49.82 ± 0.08	Crude protein (% w/v)	4.43 ± 0.05

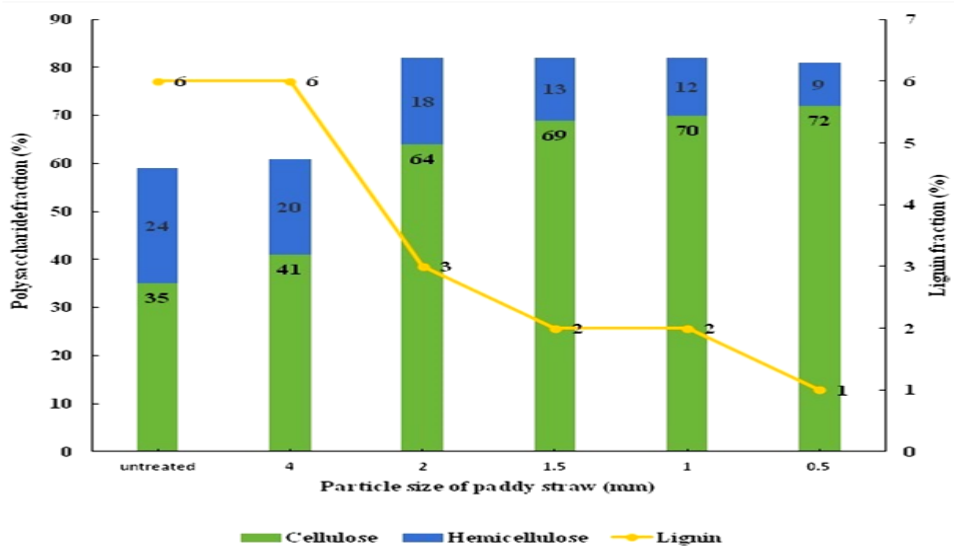

Fig. 1: Composition (cellulose, hemicellulose and lignin) of different particle sized paddy straw untreated and after treatment with alkali (2% NaOH)

sodium hydroxide revealed that reducing the particle size from 4.0 to 2.0 mm resulted in dissolution of about 50% lignin content (from 6.29 to 3.14%) [Fig. 1]. Further, decrease in particle size increased the delignification efficiency with maximum lignin removal at 0.5 mm mesh size along with maximum cellulose recovery (72%). The cellulose recovery was 69 and 70% with 1.5 and 1.0 mm particle size of paddy straw which was comparable with 0.5 mm particle size (72%) but the residual lignin content was almost half (1%) at this particle size (Fig. 1). Considering the minimum residual lignin content and maximum cellulose recovery, 0.5 mm particle size was selected for further hydrolytic studies. Wi *et al.* (2013) suggested that the popping pretreatment of rice straw can effectively improve downstream saccharification and fermentation for bioethanol production by increasing the surface area and pore volume of substrate. Sheetal *et al*. (2019) reported 4-5 times higher sugar recovery from pretreated paddy straw as compared to untreated straw on hydrolysis.

Fig. 2: Physical appearance of paddy straw before and after alkali treatment

The comparison of the physical appearance of paddy straw before and after pretreatment (0.5 mm) is given in Fig. 2. The pretreated straw looked bright yellow colored as against dull light brown color of the untreated paddy straw. Delignification was associated with decline in weight and powdery texture as compared to the rough texture of untreated straw.

Table 2: Ethanol production from paddy straw hydrolysate supplemented with molasses, cheese whey and urea at 35°C after 72 h

Treatments	Ethanol (mL L^{-1})	Ethanol yield (mL kg^{-1} paddy straw)
Paddy straw hydrolysate in citrate buffer + 0.3% urea	29.0	127.6
Paddy straw hydrolysate in cheese whey + 0.3% urea	30.0	132.0
Paddy straw hydrolysate in cheese whey	31.0	136.4
Molasses (5%)	19.0	83.6
Paddy straw hydrolysate in citrate buffer + molasses (5%) + 0.3% urea	51.0	224.4
Paddy straw hydrolysate in citrate buffer + molasses (5%)	49.0	215.6

Saccharification

Hydrolysis of alkali treated paddy straw in terms of total reducing sugars released over variable time period (1-4 h) is given in Fig. 3. Commercial cellulase loaded at 7.5 FPU g^{-1} substrate efficiently hydrolyzed the carbohydrates of paddy straw both in citrate buffer and cheese whey. Cheese whey accelerated rate of forward reaction as depicted by higher value of total reducing sugars released by cellulase enzyme at all the time intervals. Rate of cellulase catalyzed reaction was highest in 1st h of incubation and reached almost saturation after 2 h of incubation. About 50% of total reducing sugars were released from the delignified paddy straw in both the slurries *i.e.* citrate buffer (48.1%) and cheese whey (56.6%) after 1 h of hydrolysis. The enzyme activity was optimum at 2 h of incubation with 61.5 and 76.3% total reducing sugars release from paddy straw slurry in citrate buffer and cheese whey, respectively. An insignificant increase in total reducing sugars released at 3rd and 4th h of incubation indicated enzyme saturation which may be attributed to the unavailability of polysaccharides of paddy straw either due to cellulose crystallinity or residual lignin binding. Goel and Wati (2013b) reported the release of 75% total reducing sugars from alkali treated paddy straw in citrate buffer using commercial cellulase (7.5 FPU g^{-1} substrate) at 50°C after 2 h. Wi *et al.* (2013) reported a sugar recovery of 0.57 g g^{-1} biomass (glucose; 0.39 g g^{-1}) after 48 h using optimal doses of cellulase and xylanase enzymes loaded at 23 FPU and 62 IU g^{-1} biomass, respectively, and popping pretreatment of rice straw (15% substrate loading, w/v) which was significantly higher than that from untreated rice straw (total sugar recovery; 0.27 g g^{-1} biomass).

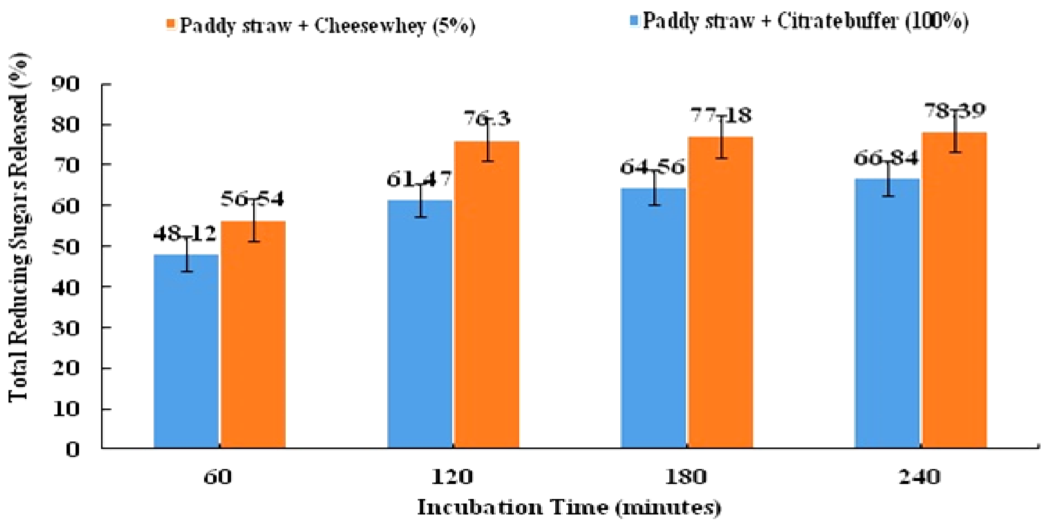

Fig. 3: Hydrolysis of alkali treated paddy straw (0.5 mm) in citrate buffer and cheese whey

Fermentation

Co-fermentation of paddy straw and cheese whey: *C. utilis* yeast strain inoculated @ 1% w/v in paddy straw hydrolysate in citrate buffer supplemented with 0.3% urea produced 29.0 mL L^{-1} ethanol corresponding to 127.6 ml ethanol kg^{-1} paddy straw. Combined fermentation of paddy straw and cheese whey under similar conditions increased ethanol production by 3.45% as observed by 30.0 mL L^{-1} ethanol production and ethanol yield of 132.0 mL ethanol kg^{-1} paddy straw. Eliminating yeast nutrients i.e. 0.3% urea further up surged the ethanol production to 31.0 mL L^{-1} ethanol and ethanol yield of 136.4 mL kg^{-1} paddy straw. Yeast nutrients supplementation had shown the suppressive effect on this combined fermentation as depicted by 3.3% increase in ethanol production value on eliminating 0.3% urea supplementation to the fermentation broth (Table 2). Priya *et al*. (2016) observed the effect of nutrient supplementation (yeast extract, $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$) on ethanol production and reported higher ethanol production from paddy straw pretreated with fungus *Myrothecium roridum* LG7, saccharified with indigenous holo-cellulase from *Aspergillus niger* SH3 without supplementation of any nutrient source. Beniwal *et al.* (2018) used silicon dioxide nanoparticle-based enzyme immobilization along with co-immobilized yeast cell and reported a maximum ethanol titer of 63.9 g L^{-1} on concentrated cheese whey (150 g L^{-1}).

Co-fermentation of paddy straw and molasses: Five percent aqueous solution of molasses on fermentation with *C. utilis* yeast strain at 35°C produced 19.0 mL L^{-1} ethanol. This corresponds to 83.6 mL ethanol kg^{-1} paddy straw. Paddy straw hydrolysate in citrate buffer under standard conditions produced 29.0 mL L^{-1} ethanol. Combined fermentation of paddy straw and molasses under similar conditions expedite ethanol production potential of yeast strain by 6.25% resulted in 51.0 mL L^{-1} ethanol production and maximal ethanol yield of 224.4 mL kg^{-1} paddy straw. Eliminating yeast nutrients i.e. 0.3% urea further decreased ethanol production to 49.0 mL L^{-1} ethanol and ethanol yield of 215.6 mL kg^{-1} paddy straw. Yeast nutrients have shown the additive effect in this co-fermentation process as observed by about 3.92% decrease in ethanol production in the absence of 0.3% urea (Table 2). Sharma *et al*. (2018) isolated two xylose fermenting *Kodamaea ohmeri* yeast strains from *Lagenaria siceraria* flowers and observed stimulated co-fermentation potential of both the strains on medium engineering with 0.1% yeast extract and peptone in rice straw fermentation. Brar *et al*. (2019) also reported co-utilization of composite sugars derived from molasses and enzymatic hydrolysate obtained from lignocellulosics like acid pre-treated rice straw, corn residues and sugarcane bagasse at higher substrate loading rates (20% w/v).

Table 3: Chemical composition of hydrolysate after fermentation

Characteristics	Value (%)
pH	5.0
Total nitrogen	3.0
Crude protein	20.0
Residual cellulose	13.0
Total reducing sugars	10.0

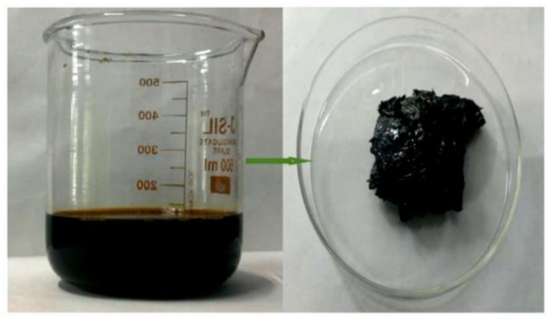

Fig. 4: Dried lignin residue extracted from pre-treated solution

The fermented residue after fermentation was analyzed for total nitrogen, crude protein, residual cellulose and total reducing sugars. Enzymatic hydrolysis and yeast fermentation converted most of the cellulose to ethanol but fermented residue contained about 10% unutilized sugars and 13% residual cellulose which is difficult to be hydrolyzed and used by the yeast strains. The spent residue of fermentation was rich in protein due to yeast biomass and can be used as feed (Table 3). The dissolved lignin can be extracted from the pre-treatment solution (Fig. 4). The dried lignin residue can be used as adhesive for industrial purpose. Saritha *et al.* (2013) reported the recovery of almost 25% depolymerized lignin as value-added acid-precipitable polymeric lignin (APPL) as compared to the control treatments.

Conclusion: The study revealed the successful combined fermentation of paddy straw (an agricultural residue) with cheese whey (by-product of cheese industry) with 6.9% expedition without yeast nutrient supplementation whereas co-fermentation of paddy straw with molasses (by-product of sugar industry) resulted in 6.25% increase in ethanol production with yeast nutrient supplementation.

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