Optimization of Attributes and Parameters for Bioethanol Production from Agricultural and Food Process Wastes

DEBASISH SAHOO [*1], VIRENDRA VAISHNAV[2], DR.TANUSHREE CHATTERJEE[3], DR.NAVITA GUPTA[4], RAJAT KUMAR NAYAK[5], and MD. IMRAN AHMAD[6]

[1*,2] *Department of Biotechnology, CSVTU, Chhattisgarh, India*
[3] *Department of Biotechnology, RITEE, Chhattisgarh, India*
[4] *Department of Life Science, BBMKU, Jharkhand, India*
[5] *Research Associate, niTza Bioventure, Odisha, India*
[6] *DBT-BITP Trainee, CRDC, Bharat Petroleum Corp. Ltd, India*

Abstract

The after-harvest waste from agricultural fields and the food process waste after their commercial utilization possess threats to environment and create various health hazards because of mismanagement, and lack of knowledge and technology. These agro waste when managed properly can be sources of energy, feed, fodder, substrate for digester, agricultural practices such as biofertilizers and bio-fillings without affecting the natural microbial and biotics of the environment. The best, efficient, effective, optimal and green utilization of these wastes is to convert them into energy. The agro waste feed stock contains large sources of carbohydrates (such as cellulose hemi-cellulose lignin and proteins) that can be explored for conversion of these stock chemical bonds into clean energy such as bio-ethanol. The renovation of agro wastes biomass into biofuels can increase fuel flexibility and reduce dependency on petroleum-based transportation fuel systems thereby reducing environmental pollution and enhancing sustainable waste management system.

Keywords: Agriculture waste, bioethanol, clean energy, environmental pollution, food process waste, waste management

Introduction

Agricultural wastes, post-processing of agricultural produce, and fruit wastes possess a great environmental concern and pollution. These wastes account to about more than 1000 metric tonnes which are mismanaged and ultimately leads to littering, incarnation, landfills, and municipal wastes causing health and environmental hazards along with pollution (Perlack, 2005). The better way of utilizing these wastes are by converting these carbon sources into green fuel (Bhatia et al., 2012). Studies by (Wheals et al., 1999) ; (Perlack, 2005) showed that many developed and developing nations such as USA, Russia, Japan, China, Brazil have adapted bio-ethanol as an alternative to fossil fuel. Proper strategies for collection, distribution, and segregation of the agro waste/food process waste with technological advancement in processing and optimization of attributes will play a vital role in the conversion of these stock carbon sources into green hydrocarbon (Hari Krishna and Chowdary, 2000) (Gould, 1984).

Post-harvest wheat straw is a prominent agro waste which approximately produce more than 185 x 10^6 tonnes globally (Ballesteros et al., 2006). India also produces huge quantity of wheat straw (Chandel and Sukumaran, 2017). Post-harvest sugarcane waste and post-process sugarcane bagasses (cane to sugar processing) are also promising agro wastes that can be utilized as substrate for bio-ethanol production (Sánchez, 2009); (Kapoor et al., 2007). Sugar cane bagasses have been used for production of 2^{nd} generation ethanol (Kapoor et al., 2007) (Betancur and Pereira Jr, 2010). Sugarcane bagasses account to more than 380 x 10^6 tonnes globally (Sánchez, 2009). Many food-processed wastes were produced from after-commercial process such as exotic fruits like pineapple, sweet lemon and litchi which are also produced in large quantities. India is the largest producer of pineapple which is one of the most consumed and processed food resources. However, there is approximately 60%-70% yield of the food process waste of pineapple reaching approximately 1527.93 x 10^3 tonnes (Bhandari et al., 2013); (Nishio et al., 1980). These wastes can be potential sources of substrate for bio-ethanol production (Hansen et al., 2013) ; (Ban-Koffi and Han, 1990). Several characteristics of sweet orange peels (un-utilized lignocellulose content after food process) such as availability, high cellulose content, and no competition with the food chain makes it an ideal substrate for bioethanol production (Braddock et al., 1999); (Castello et al., 2010); (Rani et al., 2009). India is also the largest producer and consumer of litchi fruit (National Horticulture Board, New Delhi, 2005); (Jiang et al., 2003). The high cellulose content in peels of the litchi fruit can be potential agro-waste substrate for bio-ethanol production (Sivakumar et al., 2007).

Methodology

Collection of raw material (agricultural and food process waste).

The raw material such as sugarcane *(Saccharum sp.)* bagasses, post-harvest dried wheat straw were collected from the agricultural fields in Madhya Pradesh, India. The peel wastes and post food process wastes of fruits such as pineapple *(Ananas comosus)*, litchi fruit *(Litchi chinensis)*, and sweet lemon *(Citrus sinensis)* were collected from local fruit vendors

[*] **Correspondence**: Debasish Sahoo; *Address*: Department of Biotechnology, RITEE, Raipur, Chhattisgarh, India *Email:* sahoodebasish3125@gmail.com ;

and juice centres.

Processing of raw material (agricultural and food process waste):

All raw materials were then dried in shade until they have reduced the moisture content to less than 15%. The raw materials were then milled in a roller mill into powder form for further processing.

Quantitative estimation of chemical attributes for raw material:

The quantitative estimation of total cellulose content (Atlanta, 1996), total hemicellulose content (Atlanta, 1996), total lignin content (Phenolic polymer) (Atlanta, 1996), total carbohydrate content by Anthrone test (Hedge and Hofreiter, 1962), total reducing sugar by Dinitrosalicyclate test were determined (Miller, 1959).

Pre-treatment of agro waste:

Pre-treatment is one of the most important steps where different physical and chemical methods are used to digest/decompose higher and complex sugar/carbohydrate moieties such as lignin (Phenolic polymer), cellulose and hemicelluloses (Carbohydrate polymer) into simpler sugar forms that help in better availability of sugar for fermentation (Carrol and Somerville 2009; Dashtban et al. 2009).

For the pre-treatment of agricultural and food process waste, and various physical pre-treatment such as steam explosion, microwave assisted pre-treatment, and solar pre-treatment were employed. For chemical pre-treatment, various acids and alkali such as 1% v/v Sulphuric acid (H_2SO_4), 10% v/v Sulphuric acidn(H_2SO_4), 1% v/v Sodium Hydroxide (NaOH), 1% v/v Nitric acid (HNO_3), 1% w/v Calcium hydroxide $Ca(OH)_2$ and with distilled water were used. For pre-treatment by alkali method, the agro wastes were treated for 48 hours whereas for the acid pre-treatment the agro wastes were treated for 120 minutes.

After the chemical treatment by acid/alkali, the treated agro wastes were then washed with double distilled water and dried in the hot air oven at 60°C-80°C to reduce the moisture content. The treatment was followed by different physical methods such as solar treatment, microwave irradiation and steam explosion as in Table 1.

Detoxification of pre-treated substrate:

The detoxification steps help in elimination of contaminants and different inhibitors that can hinder the process of fermentable sugar release and fermentation process (Hendriks et al. 2009).

First, the agro wastes (acid hydrolysate) were treated with calcium oxide for neutralization of pH. After neutralization, the whole mixture was at room temperature for about 30 minutes with moderate mixing. The mixture was then filtered in order to remove any contaminants and precipitate. Then, 2.5% of activated charcoal was added to the filtered mixture with continuous stirring. The mixture was then double filtered and the final pH was adjusted to 6.0-6.5.

$$Filter\ paper\ unit\ activity\ \left(\frac{FPU}{ml}\right) = \frac{Enzyme\ activity}{[enzyme]releasing\ 2.0mg\ glucose} \quad \text{Equation 1}$$

Determination of efficacy of delignification process of different pretreatment process:

The residue that is left behind after the pre-treatment and detoxified sample was washed thoroughly with distilled water to remove all associated chemicals. Then they were oven-dried at 70°C for 24 hours. The treated agro waste sample was then reweighed for percentage of weight loss method. The detoxified substrate was evaluated based on dry weight loss method (Ehrman, 1994).

Production of crude cellulase enzyme and determination of enzymatic activity:

The crude cellulase enzyme was produced by submerged state fermentation using modified Czapek Dox Medium using *Aspergillus niger* (MTCC 11098) with 3% (v/v) inoculum size. The production media was then incubated in shaking incubator at 120 rpm/minutes for 96 hours at temperature 30°C±0.2°C.

The fermentation media was then filtered in an aseptic condition by using Whatman No.1 filter paper. The filtrate was collected, centrifuged at 10,000 r.p.m for 20 minutes at 4°C. The supernatant contains crude cellulase enzyme.

The enzymatic activity for crude cellulase enzyme was carried out by procedure as described in the International Union of Pure and Applied Chemistry (IUPAC) guidelines. The cellulase activity was calculated in terms of "filter-paper units" (FPU) per milliliter (mL) of original (undiluted) enzyme solution (Ghose, 1987). The filter paper assay can be defined as Equation 1.

Hydrolysis or Saccarification:

The delignified sample was mixed with 50mM sodium acetate buffer (pH=5.0) in proportion of 1gm agro waste to 50mL sodium acetate buffer and sodium azide was added as preservative to limit microbial growth. The crude cellulase enzyme was added to the final concentration of 20 FPU of enzyme per gm of substrate.

The mixture was then shaken in a shaking incubator at 50°C at 120 r.p.m for 48 hours. Then the (enzyme-agrowaste) sample was centrifuged at 4000 rpm for 30 minutes to remove any unhydrolyzed substrate. The supernatant collected that contained the released sugar was analyzed qualitatively by Thin Layer Chromatography (Farag 1979) using the retardation factor of

Table 1. Different physicochemical pre-treatment of different agro wastes

SUBSTRATES	Chemical treatment	Physical Method
1. Sugarcane bagasses,	1% v/v Sulphuric acid (H_2SO_4)	Steam explosion
	1% v/v Sulphuric acid (H_2SO_4)	Microwave treatment
	1% v/v Sulphuric acid (H_2SO_4)	Solar treatment
2.Peel wastes of Pineapple	10% v/v Sulphuric acid (H_2SO_4)	Steam explosion
	10% v/v Sulphuric acid (H_2SO_4)	Microwave treatment
	10% v/v Sulphuric acid (H_2SO_4)	Solar treatment
3.Peel waste of Litchi fruit	1% v/v Sodium Hydroxide (NaOH)	Steam explosion
	1% v/v Sodium Hydroxide (NaOH)	Microwave treatment
	1% v/v Sodium Hydroxide (NaOH)	Solar treatment
	1% v/v Nitric acid (HNO_3)	Steam explosion
4.Peel waste of sweet lemon	1% v/v Nitric acid (HNO_3)	Microwave treatment
	1% v/v Nitric acid (HNO_3)	Solar treatment
	1% w/v Calcium hydroxide $Ca(OH)_2$	Steam explosion
	1% w/v Calcium hydroxide $Ca(OH)_2$	Microwave treatment
5.post harvest dried wheat straw	1% w/v Calcium hydroxide $Ca(OH)_2$	Solar treatment
	distilled water (dH_2O)	Steam explosion
	distilled water (dH_2O)	Microwave treatment
	distilled water (dH_2O)	Solar treatment

$$\textit{Percentage of saccarification}\ (\%) = \frac{\textit{Reducing sugar}\left(\frac{mg}{ml}\right)\times 0.9}{\text{Initial substrate conc.}\left(\frac{mg}{ml}\right)} \times 100 \quad \text{Equation 2}$$

different standard reducing sugars whereas the quantitative analysis of reducing sugar released was determined by DiNitro Salicyclic Acid method (DNSA method) (Miller, 1959); (Jain et al., 2020).

The percentage of saccharification was calculated by equation as described in Mandels and Sternberg 1976 (Equation 2).

Collection and maintenance of microbial culture for fermentation:

Different test microbial strains were selected for the fermentation process. *Pachysolen tannophilus, Pichia stipitis, Kluveromyces marxianus, Saccharomyces cerevisiae* were maintained on malt yeast agar media (YMA) and mucor indicus that was maintained on Potato Dextrose Agar (PDA).

Preparation of seed culture:

The seed culture was prepared in the sterilized media and inoculated by single microbial culture in each sterilized media, incubated at 28°C ± 2°C for 36 hours in a shaking incubator at 120rpm.

Simultaenous saccarification and fermentation (SSF):

The saccarification process was carried out. The fermentation process starts with addition of spore suspension into the hydrolysed/saccarified supernatant with definite concentration (v/v). Antifoaming agent was also added to reduce the frothing during fermentation (Kanagasabai et al., 2019). The filtrate along with seed culture were incubated at 30°C ± 2°C for 72 hours in a shaking incubator at 240rpm.

Determination of alcohol fermentation:

After incubation, the fermentation media was filtered using a filter paper. The filtrate was then diluted with 10x parts of double distilled water. The distillation of alcohol was done using glass distillation unit (Singh and Rangaiah, 2019). Total alcohol content was done by Potassium dichromate method (Morales et al., 2015); (Zhang et al., 2019)by first establishing a standard calibration curve of known concentration of ethanol. By putting the values of absorbance in the slope equation, the unknown concentration was established for each sample.

Optimization of attributes and determination of parameters for optimal fermentation:

The response ethanol yield (in gm/L) was studied using different factors (process attributes) such as inoculum concentration (1% to 6%), incubation time (24 hours to 96 hours) and incubation temperature (28°C to 34°C). A set of experimental designs were carried out and the ethanol yield of each design was established (Satheeskumar et al., 2015). A response surface experiment produced a prediction model to determine curvature, detect interactions among the design factors (independent variables), and optimize the process.

Batch fermentation of bioethanol in bench top fermentor:

A batch fermentation (Dombek and Ingram, 1987) is considered a closed system. Initially sterilized nutrient solution is fed to the fermentor and inoculated with microorganisms and incubation was allowed to proceed (Baeyens et al., 2015). During

Figure 1. Milled Sugarcane Bagasse

Figure 2. Milled Wheat Straw

the course of the fermentation, oxygen (in case of aerobic microorganisms), antifoam agents, and acid or base to control the pH were added (Prasertwasu et al., 2014). After the fermentation process, the fermentation media was collected and alcohol content was predicted by HPLC using standard ethanol calibration curve.

Results and Discussion

Processing of raw material (agricultural and food process waste)

The raw materials such as sugarcane bagasses, post harvest dried wheat straw, peel wastes of fruits such as pineapple, litchi fruit, sweet lemon were first collected, then were processed and milled (Figure 1-Figure 5).

Quantitative estimation of chemical attributes for raw material (agricultural and food process waste):

The different agro wastes were first analyzed by different chemical methodology as described for different attributes such as hemicellulose content (in %age) (Graph 1), cellulose content

Figure 3. Milled Pineapple Waste

Figure 4. Milled Sweet Lemon Peels

Figure 5. Milled Litchi Fruit Peels

(in %age) (Graph 2), lignin content (in %age) (Graph 3), total carbohydrate content (mg/ml glucose equivalent) (Graph 4) and total reducing sugar content (mg/ml glucose equivalent) (Graph 5).

Pre-treatment of agro waste:

Chemical and physical pre-treatment strategies were employed for delignification process. The weight loss is due to the removal of lignin (phenolic moieties). The greater the loss of weight equals more loss in lignin. The percentage of weight loss was used to compare the efficiency of pre-treatment effects on lignin removal.

The delignification process (percentage of weight loss method) for different physico-chemical method was determined. For estimation of efficient method of delignification for sugarcane bagasses, better method was 1%NaOH/Steam explosion (47.7%) (Graph 6), for the peel waste of pineap-

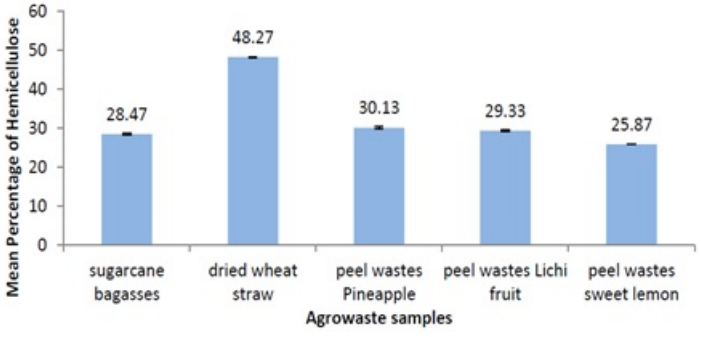

Figure 6. Hemicellulose content in agro waste samples

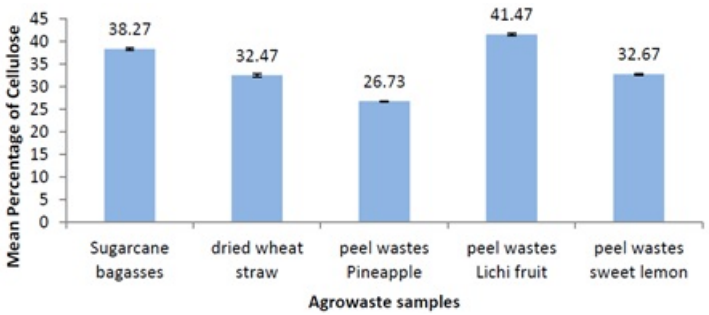

Figure 7. Cellulose content in agro waste samples

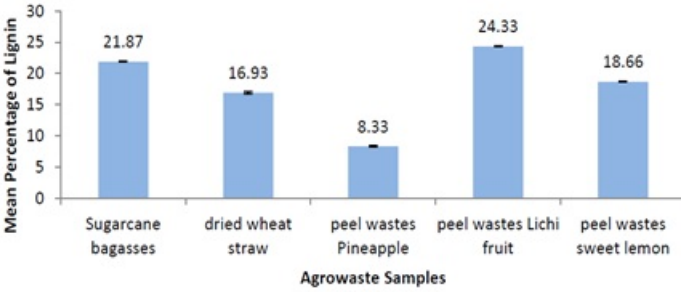

Figure 8. Lignin content in agro waste samples

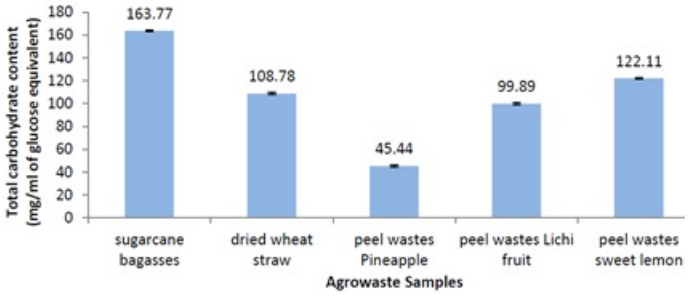

Figure 9. Carbohydrate content in agro waste samples

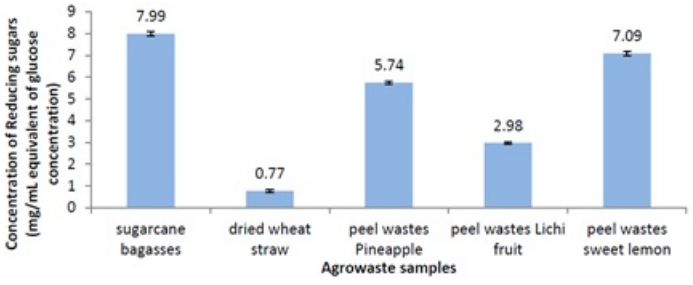

Figure 10. Reducing sugar content in agro waste samples

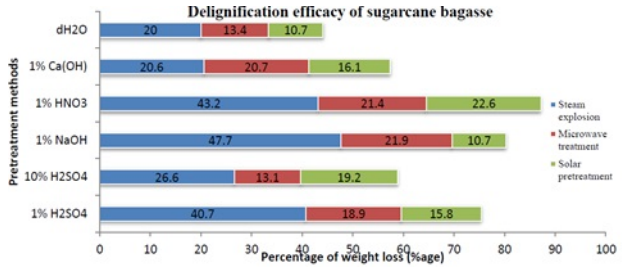

Figure 11. Assessment of delignification for sugarcane bagasse by %age of weight loss method

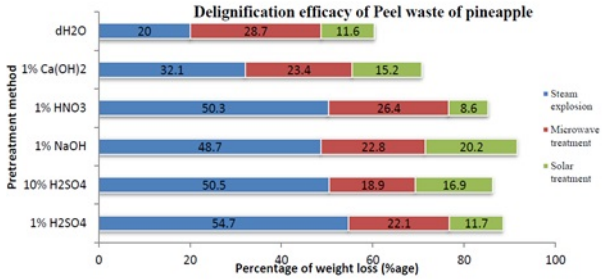

Figure 12. Assessment of delignification for peel waste of pineapple by %age of weight loss method

ple was 1%H_2SO_4/Steam explosion (54.7%) (Graph 7), for the peel waste of litchi fruit was 1%HNO_3 /Steam explosion (46.8%) (Graph 8), for the peel waste of sweet lemon was 1%HNO_3/Steam explosion (63.4%) (Graph 9), and for post-harvest dried wheat straw was 1%HNO_3 /Steam explosion (46.5%) (Graph 10).

Production of crude cellulase enzyme and determination of enzymatic activity:

The crude cellulose enzyme that has been produced by submerged fermentation was determined using *A.niger* had 31.32 FPU/mL of filter paper unit assay.

Hydrolysis or Saccarification:

The saccarification process releases reducing sugar (simpler sugar) from the higher complex sugar moieties. Thin layer chromatography method was employed to approximately

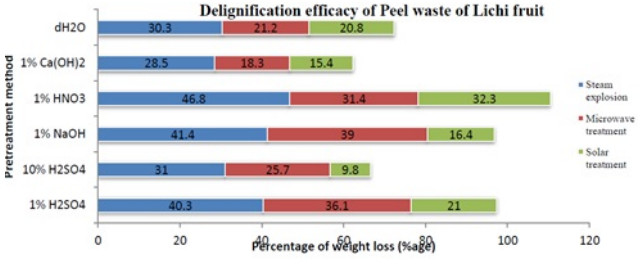

Figure 13. Assessment of delignification for peel waste of litchi fruit by %age of weight loss method

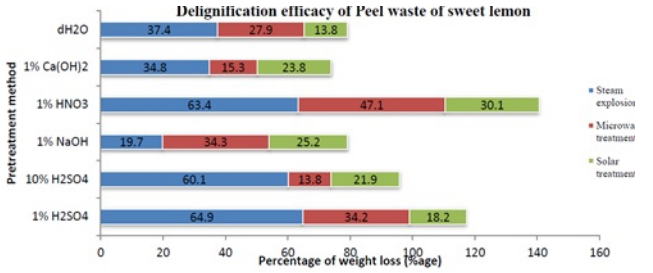

Figure 14. Assessment of delignification for peel waste of litchi fruit by %age of weight loss method

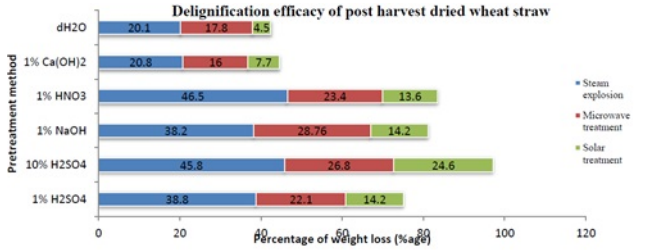

Figure 15. Assessment of delignification for post-harvest dried wheat straw by %age of weight loss method

estimate the released monosaccharides and disaccharides after saccarification process. These sugars were determined by the comparing the retardation factor of the sample as studies with the standard retardation factor to qualitatively estimate the presence of possible reducing sugars. The probable sugar released for different physico-chemical method of different agro waste, food process waste and cellulose activity were demonstrated as in Table 2 – Table 4.

The quantity of reducing sugar released (determined by DNSA method) provides the efficacy of saccarification process. The amount of reducing sugar released by employing the different physico-chemical method of pre-treatment and cellulose activity were assessed by DNSA method with amount expressed quantitatively by mg/ml glucose equivalent as demonstrated in Graph 11- Graph 13.

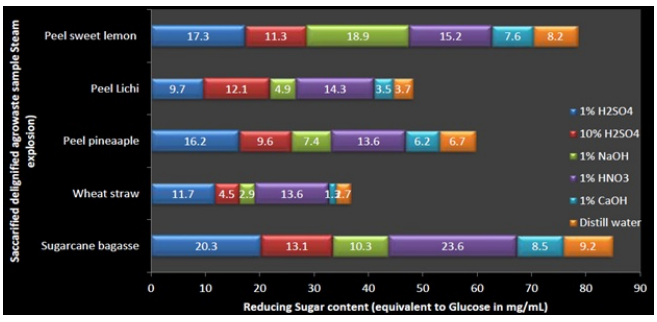

Figure 16. Total reducing sugar released after steam explosion along with different chemical pre-treatment methods for different agro waste samples

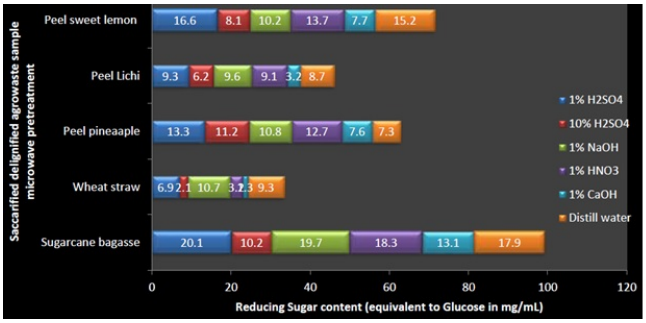

Figure 17. Total reducing sugar released after microwave pre-treatment along with different chemical pre-treatment methods for different agro waste samples

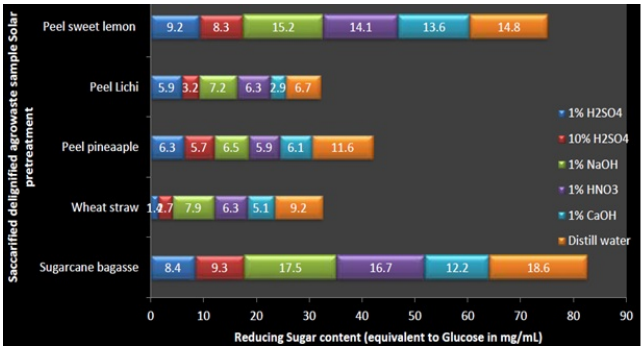

Figure 18. Total reducing sugar released after Solar pre-treatment along with different chemical pre-treatment methods for different agro waste samples

Table 2. Probable sugar released after steam explosion along with different chemical pre-treatment methods for different agro waste samples

Chemical pretreatment	Sugarcane bagasse	Dried wheat straw	Peel waste of pineapple	Peel waste of litchi	Peel waste of sweet lemon
1%v/v H_2SO_4	Xylose, Arabinose	Glucose, Fructose, Mannose	Glucose, xylose, Fructose	Glucose, mannose	Xylose, glucose
10%v/v H_2SO_4	Xylose, Arabinose	Glucose	Xylose, fructose, Mannose	Arabinose, xylose	Sorbose, Fructose
1%v/v NaOH	Sucrose, Mannose	Mannose	Glucose, mannose	Not determined	Glucose, mannose, xylose
1%v/v HNO_3	Xylose, glucose maltose	Xylose, Glucose	Arabinose, Glucose	Xylose, glucose, mannose	Arabinose, Xylose, glucose
1%w/vCa$(OH)_2$	Not determined	Not determined	Not determined	Sucrose	Not determined
Distill water	Glucose, Fructose	Not determined	Glucose, fructose, mannose	Not determined	Glucose, fructose

Table 3. Probable sugar released after microwave pre-treatment along with different chemical pre-treatment methods for different agro waste samples

Chemical pretreatment	Sugarcane bagasse	Dried wheat straw	Peel waste of pineapple	Peel waste of litchi	Peel waste of sweet lemon
1%v/v H_2SO_4	Glucose, xylose, Fructose	Not determined	Glucose	Mannose, Fructose	Glucose, Fructose
10%v/v H_2SO_4	Frucotse	Glucose, Mannose	Xylose	Not determined	Not determined
1%v/v NaOH	Glucose, Fructose	Glucose, Fructose	Not determined	Fructose, Glucose	Xylose
1%v/v HNO_3	Glucose, xylose	Glucose, xylose	Xylose, Glocuse	Glucose	Not determined
1%w/vCa$(OH)_2$	Not determined	Not determined	Glucose	Not determined	Glucose
Distill water	Glucose	Not determined	Not determined	Glucose, Fructose	Fructose

Table 4. Probable sugar released after solar pre-treatment along with different chemical pre-treatment methods for different agro waste samples

Chemical pretreatment	Sugarcane bagasse	Dried wheat straw	Peel waste of pineapple	Peel waste of litchi	Peel waste of sweet lemon
1%v/v H_2SO_4	Fructose, mannose	Not determined	Arabinose, Glucose	Xylose	Xylose
10%v/v H_2SO_4	Glucose	Glucose	Xylose	Xylose, Glucose	Not determined
1%v/v NaOH	Not determined	Glucose, Fructose	Arabinose, Glucose	Glucose, xylose	Arabinose
1%v/v HNO_3	Glucose, xylose	Xylose, Arabinose	Xylose, Arabinose	Glucose	Glucose
1%w/v$Ca(OH)_2$	Not determined	Glucose, mannose	Not determined	Rhamnose	Rhamnose, Glucose
Distill water	xylose, Fructose	Fructose	Not determined	Xylose	Glucose

Simultaneous saccarification and fermentation (SSF):

After saccarification, the filtrate for agro waste having highest amount of reducing sugar content (Graph 11- Graph 13) was selected for ethanol fermentation. The spore suspension of microbial culture such as *Pachysolen tannophilus, Pichia stipitis, Kluveromyces marxianus, Saccharomyces cerevisiae and mucor indicus*. was inoculated into the supernatant with 1.5% (v/v) with addition of antifoam agent. Fermentation parametres were managed at 30°C ± 2°C for 72 hours in shaking incubator at 240rpm.

After 72 hours, the fermentation media was centrifuged at 6000 r.p.m for 10 minutes at 4°C. The alcohol content (gm/L) in each fermentation media was determined by potassium dichromate method using calibration curve of known ethanol concentration as reference standard curve.

The highest bioethanol production from pre-treated sugarcane bagasse (1% HNO3 and Steam explosion) was from *Pachysolen tannophilus* with a total ethanol production of 9.45 mg/L, for pre-treated pineapple (1% H_2SO_4 and Steam explosion) from *Pichia stipitis* with total ethanol production of 11.58 mg/L, for pre-treated litchi fruit (1% HNO_3 and Steam explosion) from *Pachysolen tannophilus* with a total ethanol production of 10.75 mg/L, pretreated sweet lemon (1% NaOH and Steam Explosion) was from *Pachysolen tannophilus* with a total ethanol production of 12.13 mg/L and dried wheat straw (1% HNO_3 and Steam explosion) was from *Pachysolen tannophilus* with total ethanol production of 11.65 mg/L (Graph 14).

Optimization of attributes and determination of parameters for optimal fermentation:

For the design of experiment, the different response factors taken were primary inoculum concentration (1% to 6%), fermentation (incubation) time (24 hours to 96 hours) and fermentation (Incubation) temperature (28°C to 34°C). The response was ethanol yield (in gm/L). For optimal ethanol production

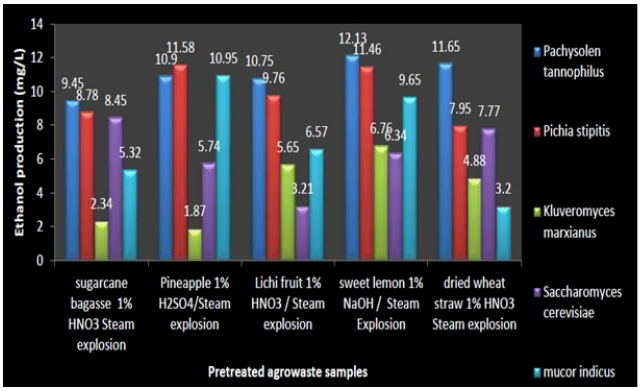

Figure 19. Ethanol yield from different agro wastes using different microbial strains for fermentation process

from different agro waste samples, again the Simultaenous Saccarification and Fermentation (SSF) was carried out and the ethanol was estimated quantitatively for each optimised agro waste fermentation conditions (Table: 5).

Batch fermentation of bioethanol in bench top fermentor:

The ethanol fermentation was carried out in bench top fermentor and was estimated quantitatively (Table 6). Highest ethanol yield with substrate as sweet lemon with Steam Explosion, 1% NaOH as physico-chemical pre-tratement method and *Pachysolen tannophilus* MTCC 1077 as the microbial strain.

Conclusion

From the above studies and review regarding the availability of agro waste raw material, pre-treatment procedures and selection of microbial strains for fermentation process, there is an ample scope and futuristic application of management of agro waste into a renewable form of energy.

Table 5. Optimization of different attributes for ethanol production from different pretreated agro-wastes

Optimized Attributes / Pre-treated Agro waste samples	Inoculum Concentration (%age) ±0.1%	Incubation Temperature (Centigrade) ±2°C	Incubation Time (Hours) ±15 minutes	pH ±0.2	Rotation Speed ±10 r.p.m.
Sugarcane Bagasse (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	5.5	32	35	5.6-5.8	240
Pineapple (Steam Explosion, 1% H_2SO_4) and *Pachysolen tannophilus* MTCC1077	1.5	30	70	5.6-5.8	240
Lichi fruit (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	4.5	28	37	5.6-5.8	240
Sweet Lemon (Steam Explosion, 1% NaOH) and *Pachysolen tannophilus* MTCC 1077	2.0	31	61	5.6-5.8	240
Post-harvest dried wheat straw (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	2.5	34	33	5.6-5.8	240

Table 6. Ethanol yield by employing optimized attributes in bench top fermentor

Sl. No.	Pre-treated agro waste and their optimized microbial culture for fermentation	Ethanol Yield (mg/ml)
1.	Sugarcane Bagasse (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	**7.45**
2.	Pineapple (Steam Explosion, 1% H_2SO_4) and *Pichia stipitis* NCIM 3498	**10.32**
3.	Litchi fruit (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	**9.11**
4.	Sweet Lemon (Steam Explosion, 1% NaOH) and *Pachysolen tannophilus* MTCC 1077	**20.72**
5.	Post harvest dried wheat straw (Steam Explosion, 1% HNO_3) and *Pachysolen tannophilus* MTCC 1077	**10.47**

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