INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS AS BYPRODUCT OF BIOETHANOL PRODUCTION FROM AGRO-WASTES

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Article Received on: 29/01/21 Approved for publication: 26/02/21

DOI: 10.7897/2230-8407.1202120

ABSTRACT

The present study aims to evaluate the potential of agro wastes such as apple pomace, Grasses, sugarcane bagasse as potential sources of bioethanol production. Bioethanol is the one of the recent increasing biofuels due to its positive impact on the environment and especially towards second generation of biofuels i.e. from non-food biomass. It's produced from high sugar and starch containing raw materials and lignocellulosic biomass. Lignocellulose may be a complex mixture of carbohydrate that needs an efficient pretreatment for the assembly of fermentable sugar, after hydrolysis are fermented into ethanol. Pretreatment of lignocellulose has received considerable research globally thanks to economic and environmental sustainability of ethanol production. Microbes like *Zymomonas mobilis,* and *Phanerochaete* provide sufficient fermentation yield and can be utilized for fermenting lignocellulosic substrate. These microbes are isolated from the feedstock samples in the present study. A suitable media was also designed for the growth of the isolated microorganisms. The antioxidant tests were analyzed on the potential samples using UV-VIS spectrophotometer. Lowering the cost of bioethanol production is one of the biggest challenges currently and can be greatly reduced by utilizing renewable feedstocks. Thus, making bioethanol is more economically competitive compared to fossil fuel.

Keywords: Bioethanol, *Zymomonas mobilis, Phanerochaete,* Pretreatment, Antioxidant test, UV-vis spectrophotometer.

INTRODUCTION

Shortage of fossil fuels and increasing heating has led to worldwide adoption of other and renewable energy sources. Energy from biomass has emerged as an environmentally friendly and sustainable solution, produced from biological sources by the fermentation process[1]. Liquid fuels like bioethanol, biobutanol, and biodiesel are being developed to replace petroleum-based transport fuels in the transportation sector. Presently the world fuel ethanol production is about 8.5 billion liters annually. From 2007 to 2008, the share of bioethanol, which was produced by fermentation process had been increased from 3.7 % to 5.4 %[2]**.** In view of current issues, such as greenhouse gas emission, fermentation and fermentation-based ethanol is considered an alternative to fossil fuel since it originates from renewable resources. The global warming is caused by using excessive fossil fuels. Therefore, renewable, clean energy and bio resource fuel are required for replacing fuel[3]. Bioethanol is a form of renewable energy that is produced from common feedstock such as agricultural waste and food wastes. In the present study, use of sugarcane bagasse, grass and apple as potential feedstock have been reported. Bioethanol production from these agricultural wastes can overcome the problem of sourcing feedstock. Production of bioethanol involves three steps: obtaining a source of fermentable sugar, conversion of sugars to ethanol through fermentation and lastly distillation process to produce ethanol. Plackett-Burman design is a type of screening design that helps to find important parameters in experiment[4]. It screens out unimportant factors thus saving huge amount of experimental time allowing to focus on only on the important parameters. This study used placket-Burman method to screen an optimized composition of fermentation media.

MATERIALS AND METHOD

Sample collection

The samples were collected nearby Hosur. Sugarcane bagasse were collected after extraction of juice from shop. Sugarcane bagasse is a common and abundant agro-waste. Grasses are ample in the environment and were collected from nearby land at Hosur. Apple pomace was collected from local shops. Pomace is a by-product of apple processing unit.

Pretreatment of biomass

10g of agricultural waste was added to 10 % HCl for acid hydrolysis and left for 30 min. Then the liquid is extracted, and sample washed with DM water. By continuous process, the sample was added to 1% of NaOH solution and left for another 30 minutes. The sample was finally washed with water after extracting the liquid. The same process was repeated for each of the other sources.

Isolation of microbes

Collected samples were used as a source for isolating microbes. The sample were serially diluted and followed by pour plate method on nutrient agar prepared by adding 0.5% peptone, 0.3% yeast extract, and 1.5% agar and kept at 34℃ incubation for 48 hr. Visible colonies were isolated using streak plate method on

CMC Agar to screen for microbes capable of producing cellulose[5]. Isolates that degraded cellulose were selected for sequencing. The cellulolytic microorganisms were identified by phylogenetic analysis.

Design of media

Suitable fermentation media for the isolated microbes, *Zymomonas mobilis* and *Phanerochaete*, were designed by placket Burman design. Among various factors only those with positive significance were selected as medium components to influence the production of bioethanol from the feed stock. The variables chosen for the present study were peptone, KH_2PO_4, K_2HPO_4, $MgSO_4$, Jaggery and NaCl for the media using *Zymomonas mobilis* for fermentation. The variables chosen for fermentation under *Phanerochaete* were dextrose and peptone. These were further optimized as Box-Behnken design[6].

Inoculation of culture to sample

Media were prepared according to the composition of the designed media. The two microorganisms were cultured in the sterilized fermentation media. These were used as inocula for the fermentation process.5 ml of the inocula were added to each of the pretreated sample under aseptic conditions. The fermentation was left to carry out for 3-4 days.

Antioxidant tests

Antioxidant tests are used to evaluate the antioxidant activity of agricultural raw materials. The constituent of each raw material varies. Therefore, to investigate antioxidant activity of chemicals, choosing an adequate assay based on the chemical is important[7]. Hereby eight antioxidant tests were used to determine various parameters such as protein, phenolic, flavonoids, total antioxidant capacity, total reducing power, free radical scavenging assay, reducing sugar, GSTAD, under UV- Vis spectrophotometer.

Determination of protein

A mixture of composition 0.2 ml of extract, 0.8 ml of water, 5ml of alkaline copper reagent was prepared and allowed to stand for 10 min. Then 0.5ml of phenol reagent was added and left for 30 min in dark and absorbance was observed at 660nm.

Determination of total phenolic

0.5ml of extract, 0.5 ml of DM water, 0.5 ml of phenolic reagent, 2g of Na_2CO_3 were taken in a test tube and mixed well. The mixture was placed dark for 40 min and absorbance was observed at 725nm.

Determination of flavonoids

0.5ml of extract, 2ml of water, 0.05 ml of Na_4N0_3 were added to make a mixture and the solution was left for 6 min. Then 0.015 g of $AlCl_3$was added and left for 6 min. Then 0.2 g of NaOH and 0.2 ml of water were added and allowed to Stand for 15min, and absorbance was observed at 510 nm.

Total antioxidant capacity

A mixture was prepared by adding 0.2 ml extract, 2ml of phenolic reagent and mixed well. It was kept at 95℃ for 90 min and left to cool for 30 min. the absorbance was observed under 695 nm.

Determination of total reducing power

1ml of extract, 1 ml of sodium phosphate buffer,1ml of potassium ferricyanide were mixed thoroughly and kept at 50℃ for 20 min. The mixture appears yellow in color. Then 1 ml of trichloroacetic acid was added. From this solution 2.5 ml is added to a mixture of 2.5 ml water and 1 ml of ferric chloride. Once the Color turns dark green, it was observed under 700 nm.

DPPH free radical scavenging assay

A solution of 30ml was prepared by adding 0.0035 g of DPPH in 30 ml IP. It was placed in Dark for 15 min and absorbance was observed at 560 nm.

Reducing sugar

On Adding the composition of 1ml extract, 1ml acetate buffer, 3ml of DNS reagent the mixture was mixed well. It was Boiled for 5min. The absorbance was measured after the mixture cooled at 440nm.

Glutathione S-Transferase activity determination

A solution of 5 ml of phosphate buffer, 1 ml EDTA and 0.2 ml PVP (Polyvinylpyrrolidone) was made. To this solution add 500µl of extract. From this sample 1ml was added to a mixture of 3ml of phosphoric acid and 2mM of reduced glutathione. The absorbance was observed under 499nm.

Table 1: *Zymomonas mobilis* media composition

Chemical Compounds	Composition (g/l)
Peptone	0.2g
KH_2PO_4	1g
K_2HPO_4	0.5g
$MgSO_4$	0.5g
Jaggery	1g
NaCl	1g

Table 2: *Phanerochaete* media composition

Chemical Compounds	Composition (g/l)
Dextrose	2g
Peptone	1g

Table 3: Test for determination of protein

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (660nm)	% of protein	Absorbance (660nm)	% of protein
Apple pomace	1.859	63%	1.899	6.2%
Sugarcane bagasse	1.861	7.1%	1.860	6.3%
Grass	1.860	6.9%	1.860	7.2%

Table 4: Test for determination of phenolics

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (725nm)	% of phenolics	Absorbance (725nm)	% of phenolics
Apple pomace	0.767	27%	0.987	27%
Sugarcane bagasse	0.703	26%	1.052	31%
Grass	0.587	21%	0.637	22%

Table 5: Test for flavonoid determination

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (510nm)	% of flavonoids	Absorbance (510nm)	% of flavonoids
Apple pomace	1.812	21%	1.822	19%
Sugarcane bagasse	1.825	24%	1.823	19%
Grass	1.826	25%	1.825	21%

Table 6: Total antioxidant capacity

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (695nm)	% of antioxidant capacity	Absorbance (695nm)	% of antioxidant capacity
Apple pomace	0.784	79%	0.689	76%
Sugarcane bagasse	0.583	58%	0.544	79%
Grass	0.582	59%	0.728	58%

Table 7: Determination of total reducing power

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (700nm)	% of reducing power	Absorbance (700nm)	% of reducing power
Apple pomace	1.756	580%	1.724	590%
Sugarcane bagasse	1.746	590%	1.752	700%
Grass	1.742	790%	1.555	790%

Table 8: DPPH free radical scavenging assay

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (560nm)	% of free radical scavenging assay	Absorbance (560nm)	% of free radical scavenging assay
Apple pomace	1.772	9.8%	1.781	10.4%
Sugarcane bagasse	1.782	10.4%	1.778	10.2%
Grass	1.782	10.4%	1.781	10.4%

Table 9: Determination reducing Sugar

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (440nm)	% of free radical scavenging assay	Absorbance (440nm)	% of free radical scavenging assay
Apple pomace	1.765	9.2%	1.777	10%
Sugarcane bagasse	1.776	9.4%	1.764	9.4%
Grass	1.767	10%	1.768	9.2%

Table 10: Glutathione S transferase activity determination (GSTs)

Samples collected	Microbes			
	Zymomonas mobilis		*Phanerochaete*	
	Absorbance (440nm)	% of reducing sugar	Absorbance (440nm)	% of reducing sugar
Apple pomace	1.846	0.49%	1.847	0.47%
Sugarcane bagasse	1.845	0.48%	1.845	0.44%
Grass	1.830	0.45%	1.846	0.45%

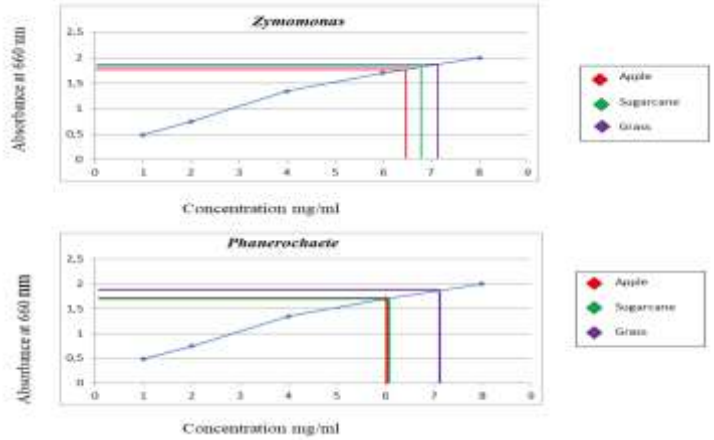

Figure 1: Estimation of protein using standard curve in *Zymomonas mobilis* and *Phanerochaete*

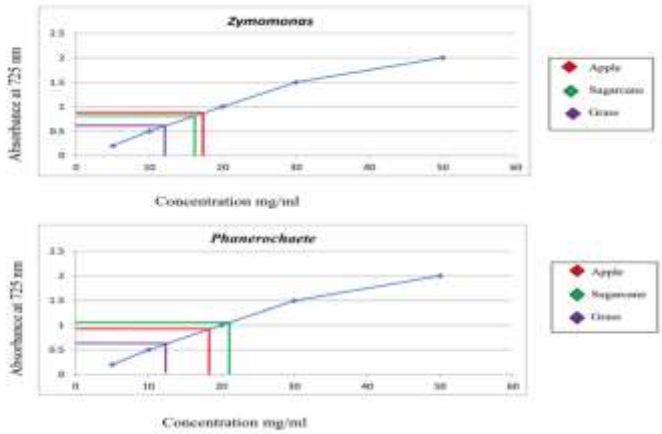

Figure 2: Estimation of phenolic content using standard curve in *Zymomonas mobilis* and *Phanerochaete*

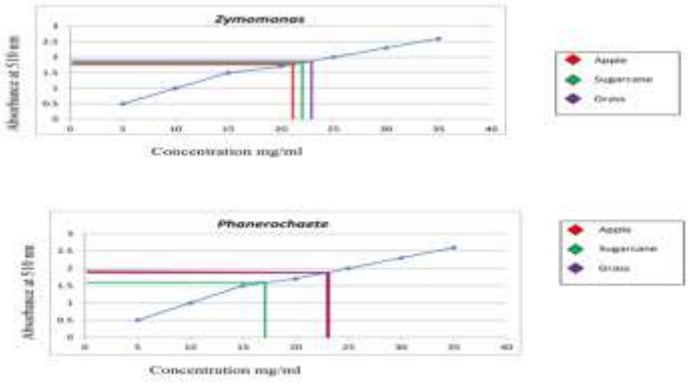

Figure 3: Estimation of flavonoid using standard curve in *Zymomonas mobilis* and *Phanerochaete*

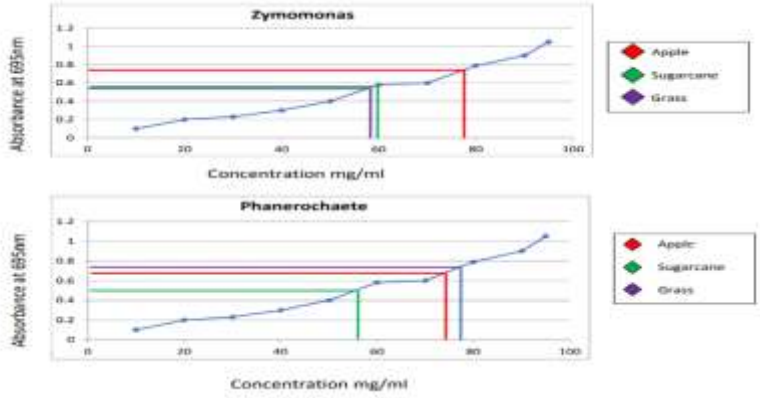

Figure 4: Estimation of total antioxidant capacity using standard curve in *Zymomonas mobilis* and *Phanerochaete*

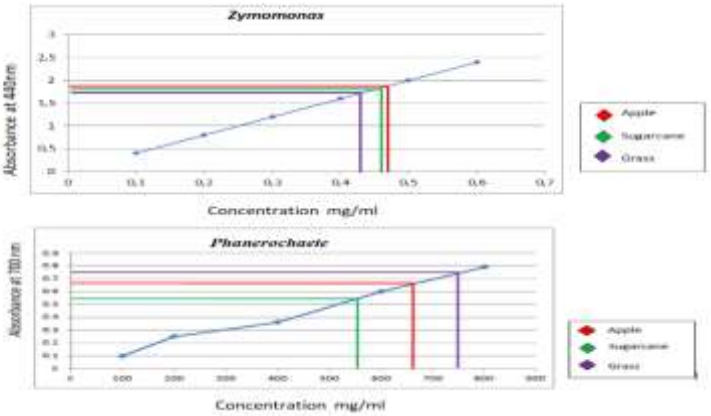

Figure 5: Estimation of total reducing power using standard curve in *Zymomonas mobilis* and *Phanerochaete*

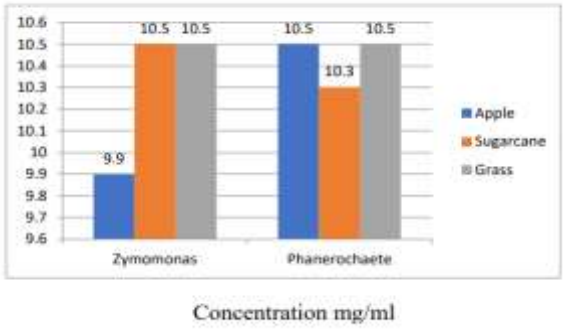

Figure 6: comparison of DPPH free radical scavenging assay at 25 µl in *Zymomonas mobilis* and *Phanerochaete*.

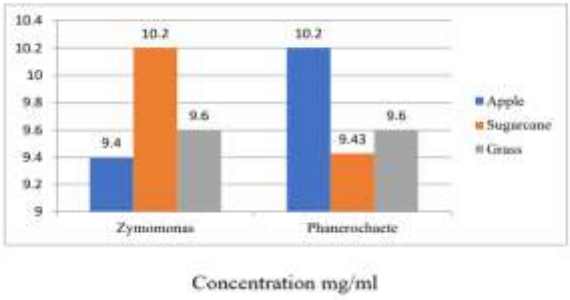

Figure 7: comparison of DPPH free radical scavenging assay at 50 µl in *Zymomonas mobilis* and *Phanerochaete*

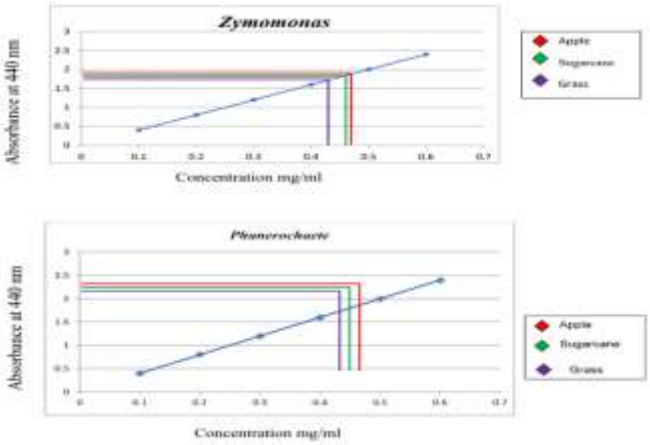

Figure 8: Estimation of Reducing sugar using standard curve in *Zymomonas mobilis* and *Phanerochaete*

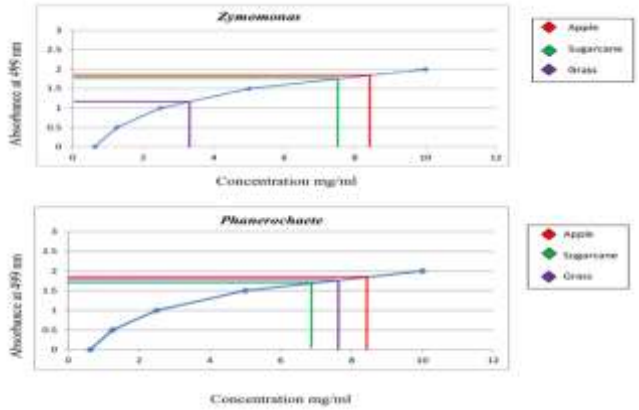

Figure 9: Estimation of Glutathione S transferase activity using standard curve in *Zymomonas mobilis* and *Phanerochaete*

RESULTS AND DISCUSSION

Pretreatment

The pretreatment of lignocellulosic biomass is to disrupt structure of converting cellulosic biomass into cellulose that is conversion of carbohydrate into fermented sugar[8]. In this study collected samples undergone Acid pretreatment followed by alkaline treatment. Acid treatment using HCl does not require enzymatic hydrolysis step, because the process itself hydrolyses the biomass to yield fermentable sugars[9].10 g of agricultural waste was added to 10% HCL for acid hydrolysis for removal of hemicellulose. By continuous process, the sample was added to 1% of NaOH solution for alkaline treatment. It helps by partially removing lignin, hemicellulose and completely removing pectin without any residue[10]. Remaining lignin content depends on NaOH concentration. Using NaOH for alkaline treatment is effective for lignocellulosic biomass[11]. The color changes observed in NaOH solution during alkaline treatment, sugarcane bagasse turns to yellow color, grass into green and apple pomace into red.

Isolation and identification of microbes

The sample were serially diluted and inoculated into agar plates and kept at 34℃ incubation for 48 hr. Visible colonies were isolated using streak plate method on sugar rich CNC media. Positive isolated showed halo zones on treatment with Congo red solution[12]. The cellulolytic strains were further sent for sequencing and identified using phylogenetic trees. The microbes were confirmed as *Zymomonas mobilis* and *Phanerochaete* by phylogenetic analysis[13].

Media designed by Plackett-Burman design

Suitable fermentation media for the isolated microbes, *Zymomonas mobilis* and *Phanerochaete*, were designed by placket Burman design[14]. Table.1 and Table.2 show the composition of the designed media for *Zymomonas mobilis* and *Phanerochaete* respectively.

Bioethanol production

The fermentation was considerably complete after 4 days. Bioethanol was produced in all the samples and by both the microorganisms. The following tests were conducted to characterize the properties of bioethanol produced.

Antioxidant tests

The antioxidant test such as total phenolics, flavonoids, capacity of antioxidant and scavenge DPPH were determined using sugarcane bagasse[15]. The presented phenolic content was 728.201± 58.21 mg, flavonoid content was 325.143±19.03 mg from the sugarcane bagasse. This study results the antioxidant test for apple pomace, sugarcane bagasse and grass. The results obtain by determination of proteins, phenolics, flavonoid, scavenge assay, reducing sugar and power, determination of catalyst and GSTs.

Determination of protein

Determination of Protein content in ethanol Produced by agro-waste samples using *Zymomonas mobilis* and *Phanerochaete*

were absorbed under 660nm using UV-vis spectrophotometer. Reading obtained were given in table 3. *Zymomonas mobilis* produced high amount of protein content in ethanol from sugarcane bagasse compared to other samples. 7.1% of protein in bioethanol was determined. *Phanerochaete* produce 7.2% of protein content in bioethanol from grass. The Overall protein content in ethanol was high in media where grass was the feedstock being fermented by *Phanerochaete* was depicted in (figure 1).

Determination of total phenolic

Determination of total phenolic in bioethanol produced through various microbes from agricultural waste was absorbed at 725nm under spectrophotometer. Reading was given in table 4. Apple pomace shows phenolic content in bioethanol as 27% under fermentation by *Zymomonas mobilis*. Whereas 31% of phenolics was determined in sugarcane bagasse under fermentation by *Phanerochaete*. Comparing both microbes *Phanerochaete* from sugarcane bagasse shows higher phenolic content of 31% in bioethanol produced by both the microorganism was shown in (figure 2).

Determination of flavonoids

Flavonoids determined in bioethanol using spectrophotometer under 510nm for various samples containing *Zymomonas mobilis* and *Phanerochaete.* Absorbance was noted in table 5. Highest flavonoid in bioethanol were determined in *Zymomonas mobilis* from grass compared to other samples. 25% of flavonoid were determined in this sample. *Phanerochaete* from grass shows 21% of flavonoids in bioethanol. On comparing both microbes, *Zymomonas mobilis* in grass shows high flavonoids content of 25% were indicated in (figure 3).

Total antioxidant capacity

Total antioxidant capacities in bioethanol produced from samples using various microbes were absorbed under 695nm and readings were noted in table 6. Samples fermented by *Zymomonas mobilis* show high antioxidant capacity of 79%. Antioxidant capacity of samples that used *Phanerochaete* in sugarcane bagasse is 79%. Highest antioxidant capacity in bioethanol is determined high in both *Zymomonas mobilis* and *Phanerochaete* in different samples were shown in (figure 4).

Determination of total reducing power

Determination of total reducing power in bioethanol were determined using spectrophotometer under 700nm. Absorbance was noted in table 7. *Zymomonas mobilis* produce high amount 790% of reducing power in bioethanol from grass. *Phanerochaete* shows 790% of reducing power in bioethanol produced from grass is determined. Both microbes show equal amount of reducing power in ethanol.

DPPH free radical scavenging assay

DDPH free radical scavenging assay were determined by measuring 25µl and 50µl using UV-vis spectrophotometer under 560nm and following table contains the noted absorbance table 8. Free radical scavenging assay in bioethanol using 25 µl sample results 10.4% from *Zymomonas mobilis* in sugarcane bagasse and grass. Highly determined free radical assay in bioethanol about 10.4 % in apple pomace and grass using *Phanerochaete*, were depicted in (figure 5). From 50 µl of sample free radical scavenging is determined high in *Zymomonas mobilis* at10% in grass. *Phanerochaete* from apple pomace is high in determining free radicle scavenging assay in bioethanol. Both microbes show same percentage of free radical scavenging assay in bioethanol of various samples such apple pomace and grass were indicated in (figure 6).

Reducing sugar

Reducing sugar in bioethanol is determined using spectrophotometer under absorbance at 440nm with *Zymomonas mobilis* and *Phanerochaete* readings are in following table 9. Reducing sugar determined in bioethanol produced by *Zymomonas mobilis* is high in apple pomace at 0.49%, 0.47% of reducing sugar is found in bioethanol from *Phanerochaete* in apple pomace. *Zymomonas mobilis* in apple pomace shows the highest at 0.49% in reducing sugar test of bioethanol were represented in (figure 8).

Glutathione S Transferase activity determination

Determination of Glutathione S transferase activity in bioethanol using spectrophotometer at 499nm absorbance for microbes used and reading were noted in table 10. Glutathione S transferase activity in bioethanol is high in apple pomace using *Zymomonas mobilis* at 8.1%. apple pomace fermented by *Phanerochaete* contains 9% of Glutathione S transferase activity in ethanol produced. On comparing *Phanerochaete* shows high determination of Glutathione S transferase in apple pomace were depicted in (figure 9).

CONCLUSION

The study shows the competence of common agricultural wastes sugarcane bagasse, apple pomace and commonly found grass as feedstock for bioethanol production. Numerous studies have shown the efficiency of *Zymomonas mobilis* as the most effective bacteria for ethanol conversion from biomass. Further studies can be conducted analyzing the potential of all the commonly available agricultural wastes broadening the scopes for second generation of biofuels.

REFERENCES

1. Aditiya HB, Mahlia TM, Chong WT, Nur H, Sebayang AH. Second generation bioethanol production: A critical review. Renewable and sustainable energy reviews. 2016 Dec 1; 66:631-53.
2. Chandra PB, Venkatesh RD, Sunil S, Kakkeri S. A review on production of ethanol from sugarcane molasses & its usage as fuel. Int. J. Mechan. Eng. Technol. 2018; 9:7-24.
3. Sarkar N, Ghosh SK, Bannerjee S, Aikat K. Bioethanol production from agricultural wastes: an overview. Renewable energy. 2012 Jan 1;37(1):19-27.
4. Braide W, Kanu IA, Oranusi US, Adeleye SA. Production of bioethanol from agricultural waste. Journal of fundamental and Applied Sciences. 2016 May 10;8(2):372-86.
5. Xu Z, Huang F. Pretreatment methods for bioethanol production. Applied biochemistry and biotechnology. 2014 Sep;174(1):43-62.
6. Amin FR, Khalid H, Zhang H, u Rahman S, Zhang R, Liu G, Chen C. Pretreatment methods of lignocellulosic biomass for anaerobic digestion. Amb Express. 2017 Dec;7(1):1-2.
7. Islam F, Roy N. Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC research notes. 2018 Dec;11(1):1-6.
8. Kavitha S, Sivamani S. Statistical optimization of cellulase production from cassava stem by Cellulomonas fimi MTCC24 using box-Behnken design. J Pharm Chem Biol Sci. 2016 Nov;4(3):375.
9.Brodeur G, Yau E, Badal K, Collier J, Ramachandran KB, Ramakrishnan S. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. Enzyme research. 2011;2011.
10. Das PK, Das BP, Dash P. Potentials of postharvest rice crop residues as a source of biofuel. In Refining Biomass Residues for Sustainable Energy and Bioproducts 2020 Jan 1 (pp. 275-301). Academic Press.

11. Dien BS, Cotta MA, Jeffries TW. Bacteria engineered for fuel ethanol production: current status. Applied microbiology and biotechnology. 2003 Dec;63(3):258-66.
12. El-Naggar NE, Deraz S, Khalil A. Bioethanol production from lignocellulosic feedstocks based on enzymatic hydrolysis: current status and recent developments. Biotechnology. 2014;13(1):1-21.
13. Ire FS, Ezebuiro V, Ogugbue CJ. Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse. Bioresources and Bioprocessing. 2016 Dec;3(1):1-2.
14. Janani K, Ketzi M, Megavathi S, Vinothkumar D, Ramesh Babu NG. Comparative studies of ethanol production from different fruit wastes using saccharomyces cerevisiae. International Journal of Innovative Research in Science, Journal Engineering and Technology. 2013 Dec;2(12):7161-7.
15. Dhanaseeli PB, Balasubramanian V. Studies of ethanol production from different fruit wastes using Saccharomyces cerevisiae. Biosciences, Biotechnology Research Asia. 2014;11(Special edition):19-23.

Cite this article as:

V. Manivasagan *et al*. Antioxidant activity of phenolic compounds as byproduct of bioethanol production from agro-wastes. Int. Res. J. Pharm. 2021;12(2):11-18.
http://dx.doi.org/10.7897/2230-8407.1202120

Source of support: Nil, Conflict of interest: None Declared