Investigation on the production of bioethanol from black tea waste biomass in the seawater-based system

Article *in* Bioresource Technology Reports · November 2018

DOI: 10.1016/j.biteb.2018.11.003

CITATIONS
30

READS
998

7 authors, including:

Indira Dash
National Institute of Technology Rourkela
14 PUBLICATIONS **269** CITATIONS
SEE PROFILE

Bhaskar Das
National Institute of Technology Rourkela
34 PUBLICATIONS **1,222** CITATIONS
SEE PROFILE

Harshita Bhawsar
Central University of Gujarat
1 PUBLICATION **30** CITATIONS
SEE PROFILE

Moumita Sahoo
National Innovation Foundation- India
16 PUBLICATIONS **301** CITATIONS
SEE PROFILE

Investigation on the production of bioethanol from black tea waste biomass in the seawater-based system

Dash Indira, Bhaskar Das, Harshita Bhawsar, Sahoo Moumita, Eldin Maliyakkal Johnson, P. Balasubramanian, R. Jayabalan

PII: S2589-014X(18)30113-0
DOI: https://doi.org/10.1016/j.biteb.2018.11.003
Reference: BITEB 112

To appear in: *Bioresource Technology Reports*

Received date: 27 August 2018
Revised date: 14 October 2018
Accepted date: 10 November 2018

Please cite this article as: Dash Indira, Bhaskar Das, Harshita Bhawsar, Sahoo Moumita, Eldin Maliyakkal Johnson, P. Balasubramanian, R. Jayabalan , Investigation on the production of bioethanol from black tea waste biomass in the seawater-based system. Biteb (2018), https://doi.org/10.1016/j.biteb.2018.11.003

Investigation on the production of bioethanol from black tea waste biomass in the seawater-based system

Dash Indira[1], Bhaskar Das[2], Harshita Bhawsar[1], Sahoo Moumita[1], Eldin Maliyakkal Johnson[1], P. Balasubramanian[2], R. Jayabalan[1*]

[1] Food Microbiology and Bioprocess Laboratory, Department of Life Science, [2] Department of Biotechnology and Medical Engineering, National Institute of Technology, Rourkela 769008, Odisha, India.

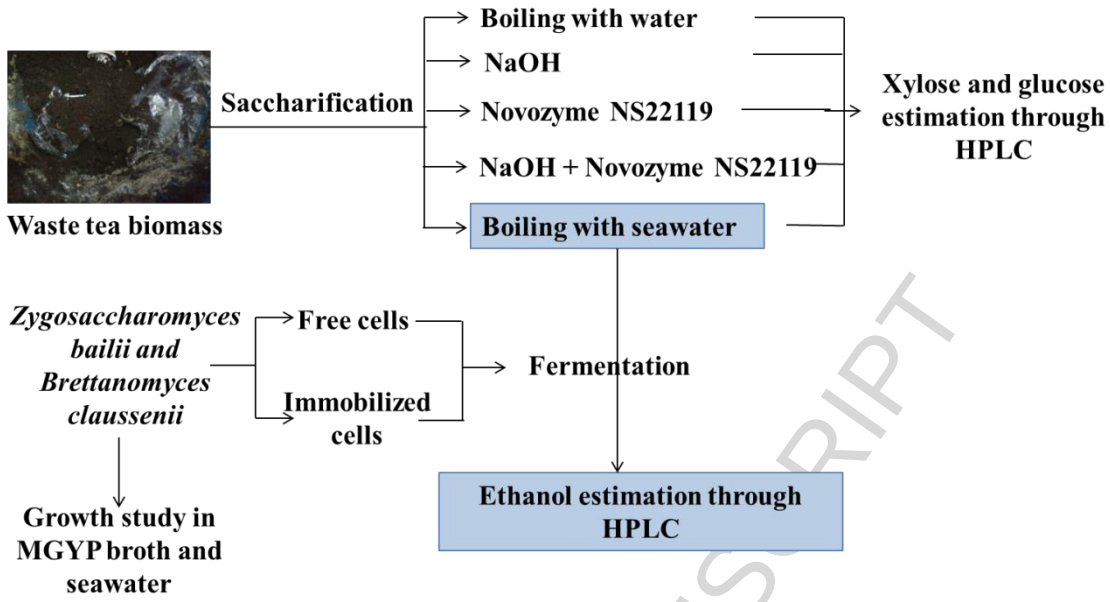
*Corresponding author, E-mail: jayabalanr@nitrkl.ac.in (R. Jayabalan)

ABSTRACT

The present study is aimed to utilize the seawater-based system for the production of bioethanol from black tea waste generated after the first brewing to reduce the consumption of freshwater in bioethanol industries. Two yeast isolates (*Zygosaccharomyces bailii* MTCC 8177 and *Brettanomyces claussenii* MTCC 7801) from kombucha black tea were evaluated for the ethanol production due to their growing capability in black tea brew as well as in seawater. The results revealed that the boiling process releases higher fermentable sugars from black tea waste than the other pretreatment methods studied. Among the two yeasts (*Zygosaccharomyces bailii* and *Brettanomyces claussenii*) studied for bioethanol production in the seawater-based medium, immobilized cells of *Z. bailii* had six-fold greater yield than the free cells. In the case of *B. claussenii* cells, there is no significant difference in ethanol yield between the free and immobilized cells on glucose fermentation.

Keywords: Kombucha black tea waste, fermentation, bioethanol, seawater, resource recovery, waste to wealth

Graphical Abstract



1. Introduction

Water plays a significant role in the energy production, extraction of sugars and in the fuel processing. The demand for fresh water is expected to increase due to the growing demands for an alternative source of energy from renewable sources (Wu et al., 2009). According to the Energy Independence and Security Act, U.S. is committed to producing 36 billion gallons of biofuel by 2022 and for which the production rate is increasing at an unprecedented rate crossing the record of 9.0 billion gallons of ethanol in 2008. Water is consumed in biofuel production mainly for growing the energy crops and processing of biofuel. Water for growing the plants yielding fermentable is supplied either by precipitation or irrigation. Replacing the sugar-rich crops with lignocellulosic biomass has reduced the water consumption in biofuel industries. Reports from U.S. Department of Energy (DOE) and U.S. Department of Agriculture (USDA) states that there is an availability of more than a billion tonnes of biomass for fuel production (Perlack et al., 2005). Aden (2007) reported that 9.8 litres of water are required for the production of one litre of ethanol from biomass and the same is estimated to reduce to 5.9 litres with an increase in ethanol yield. Global climate change and

increasing demand for fresh water are expected to pose a serious threat to the freshwater availability. Gerbens-Leenes et al. (2012) reported that the requirement of freshwater for biofuel production might increase by 5.5% by 2030 applying an additional load on scanty freshwater resources. Seawater-based biorefinery strategy is seen as an alternative to the freshwater-based biorefinery and is expected to create a considerable impact in these areas aiming at small carbon footprint, more efficient and cost-effective processes. Seawater was successfully used in enzymatic hydrolysis of lignocellulosic biomass (Grande et al., 2012) and for fermentation using halotolerant yeasts (Senthilraja et al., 2011). Urano et al. (2001) isolated marine yeasts and tested their fermentation capacity in sea water. Utilization of seawater for biofuel production reduces stress on freshwater resources while enabling the cultivation of biomass, saccharification, and processing of biofuel over a common platform. Goncalves et al. (2015) reported the production of 0.5 g ethanol per gram of glucose in seawater based media using *S. cerevisiae*.

Tea, an infusion prepared by brewing the dried green tea leaves or black tea powder is the highest consumed beverage in the world next to the water. Black tea is mostly produced from green tea leaves through CTC (Crush, Tear, Curl) process in tea industries. During manufacturing the black tea, a voluminous fibrous waste is produced which is called as tea manufacture waste or tea waste (Jayabalan et al., 2007). Tea manufacture waste produced in tea industries should not be confused with the black tea waste or spent tea waste which is left over after brewing the black tea from black tea powder in homes and tea shops. Black tea is consumed by a higher number of people than green tea due to its low cost and abundant availability. India is the second largest producer of tea in the world and has the largest number of tea consumers. In India, black tea is always consumed along with milk and sugar which are added to enhance the taste. The name black tea in India represents the infusion of

black tea powder in hot water prepared by boiling the black tea powder along with sugar and milk. During this process, extractives are being extracted, sugar is being dissolved, and the solution mixture is boiled which in turn has a pleasant flavour and mild brown. Tea is prepared in every Indian's home and the black tea powder once used (after first time brewing) is just thrown as waste. Black tea powder is reported to have 4% of carbohydrates and not all the 4% is extracted in the first time brewing which is happening in the home. The sugar added to the tea for enhancing the taste differs from home to home as per the taste variations of the individuals. The added sugar is available as a coating on the black tea waste, and it can be easily released by treating the waste in the water. The infusion prepared from the black tea waste is expected to have unextracted carbohydrates present originally in the black tea powder due to the sugar coating formed during the boiling of tea. These sugars could be fermented to ethanol. However, the challenge remains in the presence of the catechins, caffeine, theaflavins and thearubigins in black tea infusion (Jayabalan et al., 2007). The fermenting agent used for fermentation of sugars available in tea infusion must be able to grow in the same by resisting the presence of components mentioned above.

To our knowledge, there are very few reports available for the conversion of reducing sugars available in black tea waste biomass/spent tea waste and tea processing waste/tea manufacture waste/factory tea waste to biofuels (Germec et al., 2016; Mahmood et al., 2010). The present investigation was planned to explore the potency of yeasts isolated from kombucha tea to produce bioethanol by fermenting the sugars present in infusion prepared from black tea waste originated from home. Also, the present work was planned to address the reduction of fresh water in biofuel industries by replacing the fresh water in the fermenting medium with seawater.

2. Materials and methods

2.1. Collection of waste black tea powder and sea water

The one time used (brewed) waste black tea powders were collected from hostels (KMS and SD Halls of residence) and a tea shop of National Institute of Technology, Rourkela, Odisha, India. One black tea waste sample collected from a home in Koel Nagar region, Rourkela, Odisha, India was also included in the study. Sugar and milk were utilized to prepare the black tea in all the samples collected from hostels, tea shop and home. These samples represent the real scenario of the black tea waste produced in India. Also, the black tea waste prepared in the lab by one-time brewing of commercial black tea powder (Taj Mahal, Brooke Bond, India) without sugar and milk was also utilized as biomass. Seawater collected from Gopalpur, Odisha (19.27°N, 84.92°E) in June 2016 was utilized in the present study after filtering through a 0.45 µm membrane filter (HAWP04700, Millipore, India).

2.2. Yeast strains

Zygosaccharomyces bailii MTCC 8177 and *Brettanomyces claussenii* MTCC 7801 were utilized in the present study since they are isolated from tea fungus (a consortium of acetic acid bacteria and yeasts, used to produce kombucha tea) by Jayabalan et al. (2007, 2008a,b, 2014). The yeast strains were maintained in MGYP (Malt extract 1%, glucose 1%, yeast extract 0.3%, peptone 1%) medium at pH 6.0 by growing them at 30°C for 48 h.

2.3. Study of the growth potential of yeast cells in MGYP broth and tea infusion prepared with seawater

Growth potential of yeast cells (*Z. bailii* and *B. claussenii*) were studied in MGYP broth and tea infusion prepared with seawater. The MGYP broth was prepared in seawater and autoclaved as per the section 2.2. To prepare the tea infusion in sea water, 2.5 grams of commercially available black tea powder (Taj Mahal, Brooke Bond, India) was boiled for 5

min in 100 mL seawater and was filtered through Whatman filter paper. To the filtered tea broth, 0.4% $(NH_4)_2SO_4$, 0.4% KH_4PO_4, 2% $MgSO_4$, and 1.6% yeast extract were added, and pH was adjusted to 6.0. The broth was autoclaved at 121°C for 15 min at 15 lbs. *Z. bailii* and *B. claussenii* cells were inoculated into MGYP broth, and tea infusion broth prepared with seawater and incubated at 30°C for 72 h. Sampling was done at every 24 h interval. The number of yeast cells was counted by spread plate method, and colony forming units per millilitre of broth were calculated by using the following formula:

Colony Forming Units / mL = Number of cells counted / dilution factor x amount of broth taken for the analysis

2.4. Saccharification of black tea waste biomass

One time brewed black tea powder samples were thoroughly dried overnight in the hot air oven at 60ºC. The dried black tea biomass was taken for saccharification process by boiling, alkali treatment (1% NaOH), enzyme treatment (Novozyme NS22119, Denmark), and combined action of alkali and enzymes treatment. Novozyme NS22119 (Denmark) is an enzyme cocktail containing a broad range of carbohydrases.

2.4.1. Boiling with water

From the dried tea biomass, 10 g of a sample is weighed, and a tea infusion was prepared in 100 mL of distilled water by boiling the tea biomass in water for 5 min. The infusion was strained using a muslin cloth, and the extract was stored for further studies at 4ºC. The sugar content was estimated by High-Performance Liquid Chromatography (HPLC).

2.4.2. Treatment of biomass with 1% NaOH

The dried black tea waste (10 g/100 mL of distilled water) was treated with 1% NaOH and subjected to the autoclave at 121°C, 15 lbs for 20 min followed by cooling down to the room temperature. The sugar content was estimated by HPLC.

2.4.3. Treatment of biomass with Novozyme (NS22119)

Novozyme NS22119 is a cocktail of enzymes having a range of carbohydrases which includes cellulose, beta-glucanase, hemicellulose, pectinase (pectin lyase, pectin esterase and rhamnogalacturonase), arabinose, galactanase, and xylanase. The main activities of NS22119 include beta-glucanase, mannanase and polygalacturonase. As per the manufacturer's instruction, it has 100 fungal beta-glucanase units and approximately 13,700 polygalacturonase units per gram of enzyme. To 100 mL of tea infusion made from 10 g of tea powder, 50 µL of Novozymes (NS22119) was added and incubated for 48 h at 27°C. Samples were collected after 48 h to check the enzyme efficiency regarding sugar production using HPLC.

2.4.4. Combined action of 1% NaOH and Novozyme (NS22119)

The dried biomass (10 g/100 ml of distilled water) was treated with 1% NaOH and subjected to the autoclave at 121°C, 15 lbs for 20 min followed by cooling down to the room temperature. Novozyme (NS22119) (50 µL) was added to the pretreated biomass and incubated for 48 h at 27°C. Samples were collected after 48 h to estimate the sugar produced using HPLC.

2.5. Characterization of biomass using HPLC for estimation of saccharification product

Filtered samples from saccharified biomass (20 µL) were injected into the injection loop of HPLC (Shimadzu, Japan) and the samples were analyzed for the presence of reducing sugars produced by saccharification of tea powder by comparing the peaks produced by

standards of glucose and xylose. The samples were analyzed using Hiplex-H column (Agilent, USA) and one mM H_2SO_4 as mobile phase with a flow rate of 0.7 mL/min and a column temperature of 60°C with refractive index detector (Shimadzu, Japan).

2.6. Immobilization of yeast cells using calcium alginate beads

Immobilization of yeast cells (*Z. bailii* and *B. claussenii*) was done as per Indira et al. (2015). Briefly, the yeast cells were cultured on MGYP broth (pH 6.0) at 30°C and 120 rpm for 48 h. Sodium alginate (3%) and calcium chloride (0.5 M) were prepared in deionized water and autoclaved (121°C and 15 lbs) for 15 min. Yeast cells were added in 1:1 (v/v) to sodium alginate solution. The homogenized sodium alginate solution with yeast cells was taken in a syringe having a needle diameter of 1 mm and added dropwise to calcium chloride solution. The formed beads were hardened for 10 min and then washed twice with deionized water. The beads are stored aseptically for further studies.

2.7. Production of bioethanol from waste black tea biomass infusion prepared in seawater

The collected waste tea biomass sample from KMS hall of residence (as mentioned in section 2.1) was dried overnight in a hot air oven at 60°C. Infusion of waste black tea biomass was prepared by taking the waste biomass in 1 L sea water and boiled for 5 min. The tea infusion so prepared was filtered properly using Whatman filter paper. The filtered tea extract was mixed with 1 L of the seawater containing yeast extract (16.0g), $(NH_4)_2SO_4$ (4.0 g), $MgSO_4$ (20.0g), KH_4PO_4 (4.0 g) and the pH was adjusted to 6.0. The fermentation media was autoclaved at 121°C for 15 min at 15 lbs. *Z. bailii* and *B. claussenii* (immobilized and free cells, 10%) was added to the autoclaved fermentation media and incubated for 168 h at 30°C with 120 rpm. After incubation, the free and immobilized cells were filtered, and the crude broth was analyzed for ethanol content as per section 2.8.

2.8. Estimation of ethanol production by HPLC analysis

The amount of ethanol in the crude broth was estimated by HPLC (Shimadzu, Japan) analysis using Hi-Plex-H column (Agilent, USA) having column temperature of 60°C. Sulfuric acid (1 mM) was used as mobile phase with 0.7 mL/min as flow rate. Refractive index detector was used for the detection. The concentration of ethanol was determined by using an appropriate standard and using the following formula:

$$\text{Concentration of ethanol (g/L)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

The theoretical yield of ethanol is 0.51 g per 1.0 g of glucose, i.e. 2 moles of ethanol per mole of glucose and 0.51 g of ethanol per 1.0 g of xylose, i.e. 1.67 moles of ethanol per mole of xylose. Ethanol yield (g/g) is defined as the amount of ethanol produced from per gram of sugar. Ethanol yield can be calculated by the following equation:

$$\text{Ethanol yield (g/g)} = \frac{\text{Measured ethanol in sample (g)}}{\text{Theoretical ethanol (g)}}$$

2.9. Statistical analysis

Microsoft Excel 2010 was used for the calculation of mean and standard deviation. SPSS (IBM Statistics) software version 19.0 was used for comparing the means through one-way ANOVA and mean differences were compared using Duncan's multiple range test.

3. Results and discussion

3.1. Evaluation of growth of isolated yeast cells in MGYP broth and tea infusion prepared with seawater

In one of our preliminary studies, we found that most of the yeasts were not able to grow in the tea infusion (results not shown). Hence, it was decided to utilize the yeasts which are of originated from tea fungus. The yeasts utilized in the study were isolated from tea fungus which is used to produce kombucha tea (Jayabalan et al., 2008b). The yeast cells, *Z. bailii* and *B. claussenii* were studied for their growth potential in MGYP broth and black tea infusion prepared with seawater. It was evident from Table 1 that there was a substantial increase in the number of cells in the first 24 h, which can be considered as the log phase of the growth curve. Until the end of 48 h, there was a continuous increase in cell number, after which the stationary phase was reached. A similar pattern was also recorded with the yeast cells grown in the seawater-based black tea waste infusion (Table 2). The first 24 h recorded the log phase, after which there was continuous growth in cell number. After 48 h the stationary

Table 1: Growth of yeast in MGYP medium prepared with seawater.

Time (h)	*Z. bailii*	*B. claussenii*
	Log_{10} of CFU/mL	
0	7.45 ± 0.38	7.80 ± 0.51
19	8.59 ± 0.34	8.71 ± 0.31
48	9.00 ± 0.41	9.05 ± 0.22
60	9.03 ± 0.22	9.05 ± 0.45
77	9.06 ± 0.42	9.04 ± 0.23
100	9.02 ± 0.35	9.07 ± 0.18

Values are mean + standard deviation

n=3 (cells are counted from three dilutions)

Table 2: Growth of yeast in black tea infusion prepared with seawater.

Time (h)	*Z. bailii*	*B. claussenii*
	Log_{10} of CFU/mL	
0	2.00 ± 0.23	2.00 ± 0.21
24	2.85 ± 0.25	2.81 ± 0.24
48	3.34 ± 0.33	3.10 ± 0.35
72	3.62 ± 0.42	3.37 ± 0.41
96	3.76 ± 0.33	3.40 ± 0.11
110	3.76 ± 0.41	3.51 ± 0.32
124	3.78 ± 0.21	3.80 ± 0.25
146	3.84 ± 0.28	3.83 ± 0.26

Values are mean + standard deviation
n=3 (cells are counted from three dilutions)

phase was recorded. It was evident from the Table 1 and Table 2 that yeast cells were able to grow in tea infusion prepared in seawater. From the above pattern, it is clear that the cells after 24 h of seeding are at log phase of the growth curve and therefore, 24 h old culture is best suited for inoculum purpose.

Many of the yeasts are reported to tolerate the salt stress by several mechanisms (Andreishcheva and Zviagilskaia, 1999; Goncalves et al., 2015). Yeast cells manage the salt stress by synthesizing osmolytes or compatible solutes (glycerol, polyols, trehalose, and glycogen) to balance the intracellular osmotic pressure with the external environment or by ejecting the excessive intracellular Na^+ ions by Na^+/H^+ antiporter systems (Yancey, 2005;

Kogej et al., 2007; Gostincar et al., 2008). Hence, *Z. bailii* and *B. claussenii* cells would be having these mechanisms to tolerate the salt available in seawater.

3.2. Saccharification of used black tea waste biomass

The waste black tea biomass samples collected and prepared in the lab were saccharified using variable treatment methods, i.e. boiling, alkali hydrolysis using 0.1% NaOH, enzyme hydrolysis by Novozymes (NS22119) and combined action of 1% NaOH and Novozymes (NS22119). Saccharified samples were evaluated for the release of glucose and xylose in HPLC. Glucose and xylose were released in all the samples through all the saccharification methods. The fermentable sugars released after the saccharification possibly released from the black tea waste biomass itself or from the added sucrose which would have coated on the biomass. The other source of the fermentable sugars is the lactose from milk which is usually added to prepare the tea in India. The concentration of the lactose in the tea waste biomass was not studied in the present study.

Among the samples tested, black tea waste biomass collected from SD hall of NIT Rourkela was found to release a higher concentration of glucose and xylose after different saccharification methods studied. Boiling, alkali and alkali with enzyme saccharification treatments were found to release more glucose and xylose from black tea waste biomass of SD hall than enzyme saccharification alone. Followed by SD Hall, black tea waste biomass from KMS hall of NIT Rourkela found to release a higher concentration of glucose and xylose after different saccharification treatments. However, there was no significant difference ($P<0.05$) observed among the saccharification treatments studied (Table 3).

Black tea waste biomasses collected from the shop, home and generated in the lab were found to release fewer amounts of glucose and xylose compared to SD and KMS Halls. This may be due to the usage of fewer amounts of sugars in shop and home than in institute hostels to prepare the tea for consumption. Similar to SD hall tea waste biomass, boiling, alkali and alkali with enzyme saccharification produces higher glucose and xylose than the enzyme saccharification alone from these three black tea waste biomasses also (Table 3). The release of less fermentable sugars through enzyme saccharification may be ascribed to the following reasons; usage of less amount of enzyme which is not sufficient for saccharification, the complexity of the surface of tea waste biomass which might not allow the enzyme to work on it, and the unfavourable environment/conditions for the enzyme. Hence, it can be concluded that the usage of the enzyme alone to saccharify the black tea waste biomass is not an economical procedure. Among the other three treatments studied in the present study, saccharification by boiling was found to release a higher concentration of fermentable sugars than alkali and alkali with enzyme saccharification. Though boiling is an energy-intensive process, it does not require the addition of alkali and enzyme and does not leave any chemicals in the sample which will be subsequently fermented. Due to these advantages, boiling may be

Table 3: Evaluation of the content of glucose and xylose of black tea waste biomass.

Pretreatments (Saccharification) / Samples	Glucose (%)	Xylose (%)
Black tea waste biomass from the shop		
Boiling	1.24 ± 0.02^{a}	1.70 ± 0.14^{a}
1% NaOH pretreatment	0.63 ± 0.22^{b}	0.86 ± 0.43^{b}
Novozyme hydrolysis	0.48 ± 0.032^{bc}	0.52 ± 0.022^{b}
1% NaOH + Novozyme hydrolysis	0.40 ± 0.013^{c}	1.55 ± 0.32^{a}
Black tea waste biomass from KMS Hall		
Boiling	1.67 ± 0.16^{a}	1.95 ± 0.19^{a}
1% NaOH pretreatment	1.54 ± 0.56^{a}	2.01 ± 0.81^{a}
Novozyme hydrolysis	1.20 ± 0.23^{a}	1.20 ± 0.25^{a}
1% NaOH + Novozyme hydrolysis	1.03 ± 0.23^{a}	1.35 ± 0.78^{a}

Black tea waste biomass from SD Hall		
Boiling	3.00 ± 0.32^{a}	3.66 ± 0.52^{a}
1% NaOH pretreatment	2.13 ± 0.52^{a}	2.79 ± 0.67^{b}
Novozyme hydrolysis	0.47 ± 0.03^{b}	0.52 ± 0.07^{c}
1% NaOH + Novozyme hydrolysis	3.00 ± 0.87^{a}	2.34 ± 0.23^{b}
Black tea waste biomass from home		
Boiling	0.18 ± 0.07^{a}	0.18 ± 0.03^{a}
1% NaOH pretreatment	0.14 ± 0.08^{a}	0.12 ± 0.06^{ab}
Novozyme hydrolysis	0.03 ± 0.002^{b}	0.09 ± 0.004^{b}
1% NaOH + Novozyme hydrolysis	0.16 ± 0.003^{a}	0.13 ± 0.007^{ab}
Black tea waste biomass generated in the lab		
Boiling	0.065 ± 0.008^{b}	0.09 ± 0.007^{b}
1% NaOH pretreatment	0.68 ± 0.32^{a}	0.73 ± 0.52^{a}
Novozyme hydrolysis	0.03 ± 0.002^{b}	0.08 ± 0.01^{b}
1% NaOH + Novozyme hydrolysis	0.92 ± 0.052^{a}	0.83 ± 0.06^{a}

Data represents mean ± standard deviation; n = 3.
Superscript letters which are not similar in a column are significantly different from each other between different treatments in a group ($P<0.05$).

recommended for the saccharification of the black tea waste biomass. The fermentable sugars estimated after saccharification of black tea waste biomass are comparable with the available literature. Yucel and Goycincik (2015) have reported the generation of 28.90 g reducing sugar/L after acid and cellulase mediated saccharification of spent tea waste. Their study was carried out with the optimized concentration of NH_4Cl (2.7 g/L), yeast concentration (11.7 g/L) and temperature (42.8°C). In the present study, the maximum glucose and xylose concentration observed is 3.00% (30 g / L) and 3.66% (36.6 g/L) when the tea waste biomass from SD hall of residence was subjected to boiling whereas the alkali and novezyme treatment generated 3.00% (30 g/L) and 2.34% (23.4 g/L), respectively.

3.3. Production of ethanol using waste black tea biomass infusion prepared in seawater

Fermentation of waste black tea biomass infusion prepared in seawater was carried out using both free and immobilized cells of *Z. bailii* and *B. claussenii* for 168 h, and the ethanol was measured after every 24 h of fermentation. Initial glucose and xylose concentration in the waste black tea biomass infusion was found to be 1.87% and 2.25%, respectively. From table 4, it is evident that immobilized *Z. bailii* cells performed better than other treatments studied. Immobilized *Z. bailii* cells were able to produce 1.15% ethanol with a yield of 0.54. The concentration of ethanol was constantly increasing with the increase in fermentation time. Immobilization of *Z. bailii* cells had a significant effect after 96 h of fermentation. Compared to un-immobilized cells, fermentation of waste black tea biomass by immobilized *Z. bailii* cells resulted in an increased ethanol yield to 0.24, 033, and 0.54 compared to 0.15, 0.19, and 0.20, after 120, 144, and 168 h of fermentation, respectively. In the case of *B. claussenii* cells, immobilization did not have a significant effect on ethanol production except after 168 h of fermentation. Fermentation by immobilized *B. claussenii* cells for 168 h resulted in an ethanol yield of 0.27 compared to 0.19 by un-immobilized cells. It was interesting to note that free cells of *Z. bailii* and *B. claussenii* were also able to do fermentation in the presence of tea polyphenols present in an infusion of black tea waste biomass and seawater. Free cells and immobilized cells of *Z. bailii* were found to be a better fermenter than *B. claussenii*.

Table 4: Production of ethanol by kombucha yeasts in black tea infusion prepared with seawater from black tea waste biomass (initial glucose concentration, 1.87%, and initial xylose concentration 2.25%, w/v).

Hours of Fermentation	Ethanol (%, w/v)	
	Z. bailii free cells	*Z. bailii* immobilized cells
24	ND	0.12 ± 0.055 (0.06)
48	0.22 ± 0.043[a] (0.10)	0.17 ± 0.019[a] (0.08)
72	0.27 ± 0.063[a] (0.13)	0.28 ± 0.027[a] (0.13)
96	0.24 ± 0.083[a] (0.11)	0.34 ± 0.041[a] (0.16)

120	0.32 ± 0.039^{a} (0.15)	0.51 ± 0.087^{b} (0.24)
144	0.39 ± 0.078^{a} (0.19)	0.70 ± 0.044^{b} (0.33)
168	0.44 ± 0.098^{a} (0.20)	1.15 ± 0.064^{b} (0.54)
	B. claussenii free cells	*B. claussenii* immobilized cells
24	0.19 ± 0.071^{a} (0.10)	0.11 ± 0.064^{a} (0.05)
48	0.23 ± 0.048^{a} (0.11)	0.17 ± 0.019a (0.08)
72	0.24 ± 0.043^{a} (0.11)	0.21 ± 0.028^{a} (0.10)
96	0.25 ± 0.061^{a} (0.12)	0.24 ± 0.058^{a} (0.11)
120	0.34 ± 0.081^{a} (0.16)	0.38 ± 0.087^{a} (0.18)
144	0.38 ± 0.024^{a} (0.18)	0.46 ± 0.027^{a} (0.22)
168	0.39 ± 0.055^{a} (0.19)	0.56 ± 0.098^{b} (0.27)

NA - Not Applicable; ND - Not Detected
Data represents mean ± standard deviation; n = 3 (3 measurements)
Values in parenthesis represent the ethanol yield (g/g)
Superscript letters which are not similar in a row are significantly different from each other ($P<0.05$)

Yucel and Goycincik (2015) have reported the production of 12.72 g ethanol / L by *S. cerevisiae* after 24 h of fermentation of reducing sugars from spent tea waste. Their study was carried out with the optimized concentration of NH_4Cl (2.7 g/L), yeast concentration (11.7 g/L) and temperature (42.8°C). Compared to the previous study conducted for the production of ethanol from other biomasses, the present study reports very less ethanol even after 168 h of fermentation. However, the immobilized *Z. bailii* yeast cells were able to produce 11.5 g ethanol / L after 168 h of fermentation which is highly comparable to the results of Yucel and Goycincik (2015). This slower production might be due to the presence of tea polyphenols and seawater which would have given stress to yeast and made the yeast to produce less ethanol. Tea polyphenols have been reported to be inhibitors of microbial growth and fermentation. Hydrolysis of lignocellulosic materials is known to form or release a broad range of compounds which are inhibitory to microorganisms. The inhibitors are usually divided into three main groups based on their origin: weak acids, furan derivatives, and

phenolic compounds. These compounds are found to limit the efficient utilization of the hydrolysates for ethanol production by fermentation. Palmqvist and Hahn-Hagerdal (2000) have reviewed the mechanisms of inhibitors of fermentation produced during hydrolysis of lignocellulosic biomass. Hydroxymethylfurfural, furfural, vanillin, 4-hydroxy benzoic acid, are well known phenolic inhibitors of yeast fermentation generated through hydrolysis of lignocellulosic biomass (Ranjan et al., 2009). Phenolic compounds partition into biological membranes and cause loss of integrity, thereby affecting the ability to serve as selective barriers and enzyme matrices. Phenolic compounds have been suggested to exert a considerable inhibitory effect on the fermentation of lignocellulosic hydrolysates, the low molecular weight phenolic compounds being most toxic (Palmqvist and Hahn-Hagerdal, 2000). The presence of phenolic compounds in infusion prepared from waste black tea biomass and seawater may be the reasons for the reduction in ethanol production compared to the earlier reports.

4. CONCLUSIONS

This study attempted the seawater-based system for bioethanol production from black tea waste biomass. From the above experimental data, it can be concluded that the black tea waste biomass can be pretreated by boiling to extract the fermentable sugars and then fermented with yeasts isolated from kombucha black tea in the seawater-based system. Used as a source for bioethanol production. Studies on optimization of the fermentation conditions could enhance the ethanol productivity, and further research on yeast capabilities are needed to bring the breakthrough in the field of fermentation technology.

Acknowledgements

The authors acknowledge the Ministry of Human Resources and Development, Government of India for the funding under research scholar category to Mrs. Indira Dash, Mr. Bhaskar Das, Ms. Moumita Sahoo, and Mr. Eldin Maliyakkal Johnson. Further, the authors extend their thanks to the caterers of KMS and SD halls of residences, Mr. Arun, owner of a tea shop in NIT Rourkela campus for providing the basic raw materials.

References

Aden, A. 2007. Water usage for current and future ethanol production", Southwest Hydrology, 2007, 6(5), 22-23, http://www.swhydro.arizona.edu/archive/V6_N5/feature4.pdf

Andreishcheva, E., Zviagilskaia, A. 1999. Adaptation of yeasts to salt stress. Prikl. Biokhim. Mikrobiol., 35, 243-256.

Gerbens-Leenes, P.W., Van Lienden, A.R., Hoekstra, A.Y., Van der Meer, T.H. 2012. Biofuel scenarios in a water perspective: The global blue and green water footprint of road transport in 2030. Global Environ. Chang. 22(3), 764-775.

Germec, M., Turhan, I., Yatmaz, E., Tetik, N., Karhan, M. 2016. Fermentation of acid-pretreated tea processing waste for ethanol production using *Saccharomyces cerevisiae.* Scientific bulletin. Series F. Biotechnologies, Vol. XX, ISSN 2285-1364, CD-ROM ISSN 2285-5521.

Gonçalves, F.A., Santos, E.S.D., de Macedo, G.R. 2015. Alcoholic fermentation of *Saccharomyces cerevisiae, Pichia stipitis* and *Zymomonas mobilis* in the presence of inhibitory compounds and seawater. J. Basic Microbiol. 55(6), 695-708.

Gostincar, M., Turk, T., Trbuha, T., Vaupotic, A., Plemenitas, M., Gunde-Cimerman, N. 2008. Expression of fatty-acid modifying enzymes in the halotolerant black yeast *Aureobasidium pullulans* under salt stress. Stud. Mycol., 61, 51-59.

Grande, P.M., De Maria, P.D. 2012. Enzymatic hydrolysis of microcrystalline cellulose in concentrated seawater. Bioresour. Technol. 104, 799-802.

Indira, D., Jijnasa, B., Arati, N., Moumita, S., Ajay, D., Eldin, J., Sachin, K., Jayabalan, R. 2015. Comparative studies of ethanol production and cell viability: free cells versus immobilized cells. Res. J. Pharm. Biol. Chem. Sci., 6(20), 1708-1714.

Jayabalan, R., Marimuthu, S., Swaminathan, K. 2007. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. Food Chem., 102, 392-398.

Jayabalan, R., Subathradevi, P., Marimuthu, S., Sathishkumar, M., Swaminathan, K. 2008a. Changes in free-radical scavenging ability of kombucha tea during fermentation. Food Chem., 109, 227-234b.

Jayabalan, R., Marimuthu, S., Thangaraj, P., Sathishkumar, M., Binupriya, A.R., Swaminathan, K., Yun, S-E. 2008b. Preservation of kombucha tea – effect of temperature on tea components and free radical scavenging properties. J. Agric. Food Chem., 57, 9064-9071.

Jayabalan, R. Malbasa, R.V., Loncar, E.S., Vitas, J.S., Sathishkumar, M. 2014. A Review on Kombucha Tea – Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. Compre. Rev. Food Sci. Food Safety, 13, 538-550.

Kogej, T., Stein, M., Volkmann, M., Gorbushina, A., Galinski, A., Gunde-Cimerman, N. 2007. Osmotic adaptation of the halophilic fungus Hortaea werneckii: role of osmolytes and melanization, Microbiol., 153, 4261-4273.

Mahmood, T., Hussain, S.T. 2010. Nanobiotechnology for the production of biofuels from spent tea. African J. Biotechnol., 9(6), 858-868.

Palmqvist, E., Hahn-Hagerdal, B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour. Technol., 74, 25-33.

Perlack, R.D., Wright, L.L., Turhollow, A.F., Graham, R.L., Stokes, B.J., Erbach, D.C. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of

a billion-ton annual supply. Oak Ridge National Laboratory, https://www1.eere.energy.gov/bioenergy/pdfs/final_billionton_vision_report2.pdf.

Ranjan, R., Thust, S., Gounaris, C.E., Woo, M., Floudas, C.A., Keitz, M.V., Valentas, K.J., Wei, J., Tsapatsis, M. 2009. Adsorption of fermentation inhibitors from lignocellulosic biomass hydrolyzates for improved ethanol yield and value-added product recovery. Microporous Mesoporous Mater., 122, 143-148.

Senthilraja, P., Kathiresan, K., Saravanakumar, K. 2011. Comparative analysis of bioethanol production by different strains of immobilized marine yeast. J. Yeast Fungal Res. 2(8), 113-116.

Urano, N., Yamazaki, M., Ueno, R. 2001. Distribution of halotolerant and/or fermentative yeasts in aquatic environments. J.-Tokyo Univ. Fisheries 87, 23-30.

Wu, M., Mintz, M., Wang, M., Arora, S. 2009. Water consumption in the production of ethanol and petroleum gasoline. Environ. Manag., 44(5), 981-997.

Yancey, H. 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol., 208, 2819- 2830.

Yucel, Y., Goycincik, S. 2015. Optimization of ethanol production from spent tea waste by *Saccharomyces cerevisiae* using statistical experimental designs. Biomass Convers. Biorefin., 5, 247–255.

Highlights

1. Sea water is used in bioprocesses to address the threats on depletion of fresh water.
2. Black tea waste biomass is utilized to produce bioethanol in sea water system.
3. Kombucha yeasts are studied for tolerance to tea polyphenols and sea water.
4. *Z. bailii* and *B. claussenii* produce ethanol in sea water based system.