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SUSTAINABLE UTILIZATION OF FRUIT WASTES FOR PRODUCTION OF BIOETHANOL USING THERMOTOLERANT *SACCHAROMYCES CEREVISIAE* YEAST ISOLATED FROM COMMON FRUITS OF BANGLADESH

F. Khatun[1], Z. Islam[1, 2], A. Habib[1], S. Saha[1] and S. Yasmin[1]

[1]Department of Biotechnology, Bangladesh Agricultural University, Mymensingh-2202, BANGLADESH
[2]Graduate School of advanced life sciences, Biotechnology department, Ritsumeikan University, Japan
Corresponding author's email: fahmida.meem@bau.edu.bd

ABSTRACT

Bioethanol is one of the most important biofuel due to its positive impact on the environment. However, its economic production is dependent primarily on the raw materials used and the fermentation potentiality of the microorganisms. Therefore, this study was aimed to screen for thermotolerant *Saccharomyces cerevisiae* yeast isolates from common fruits of Bangladesh and produce bioethanol from decaying fruit wastes. Nine different fruits were collected and subjected for anaerobic growth. Colonies similar to *S. cerevisiae* yeast were isolated based on cultural (colony shape, size, color, elevation, surface edge, consistency), morphological (bud, cell shape) and biochemical characteristics (carbohydrate fermentation). Molecular confirmation was done using *S. cerevisiae* RSP5-C allele specific primer. Six isolates were confirmed as *S. cerevisiae* based on cultural, morphological, biochemical and molecular characteristics studied. From the four isolates that showed the growth capacity at a higher (41 ºC) temperature than optimum (30 ^{0}C), SC-Gr, the isolate from grape, showed the most active growth at 41 ºC and used to produce bioethanol from decaying fruits like banana, papaya, apple and grape. The significantly highest amount of bioethanol concentration (7.92 %) was obtained from the grape waste at 37 ºC, at pH 5 after 48 hrs of the fermentation period and the lowest amount of bioethanol (2.37 %) was obtained from decaying banana at 45 ºC and pH 5.0 after 72 hrs of fermentation period. This study suggested that decaying fruits could be better utilized in the industry of renewable fuel energy using thermotolerant yeast isolate from grapes.

Keywords: Biofuel, Decaying fruit wastes, Fermentation, Thermotolerance, *RSP5-C* allele

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INTRODUCTION

Overuse of fossil fuels and consequent environmental issues has become a concern recently for the production of alternative fuels. Microbial conversion of renewable cellulosic and lignocellulosic feedstock to biofuel such as bioethanol is very effective to minimize the problems associated with the world environment (Cho *et al.,* 2015). Bioethanol is a sustainable energy source and clean-burning fuel, which gives higher thermal efficiency and power than conventional gasoline (Hansen *et al.,* 1985).

Yeasts, particularly the genus *Saccharomyces*, and mainly the species *Saccharomyces cerevisiae*, are usually the first choice for industrial bioethanol production because of their good fermentative capacity, higher ethanol yield, high tolerance to ethanol and other inhibitors which occur during pre-treatment or fermentation, and the capacity to grow rapidly under the anaerobic conditions that are typically established in large-scale fermentation vessels (Mussatto *et al.,* 2010; Mohd Azhar *et al.,* 2017). The yeast *S. cerevisiae* is considered as safe microorganism to produce bioethanol (Pretorius Du Toit *et al.,* 2003). Among different microorganisms, *S. cerevisiae* is the major bioethanol producer that can produce up to (20% v/v) by fermentation from different carbon sources (Cot *et al.,* 2007). However, during industrial scale production of bioethanol, some factors affect the production rate, for instance, toxicity of ethanol (Torija *et al.,* 2003), temperature sensitivity (Talukder *et al.,* 2016), distillation cost (Alfenore *et al.,* 2002) etc. Among these factors, production rate faces serious problem due to temperature sensitivity. In general, optimum growth temperature for *S. cerevisiae* is 25 ^{0}C-30 ^{0}C and growth rate decreases rapidly at above 30 ^{0}C (Torija *et al.,* 2003). However, different research works suggest that thermotolerant yeasts can survive and ferment ethanol at above 30 ^{0}C (Suutari *et al.,* 1990; Banat *et al.,* 1992; Nonklang *et al.,* 2008). Bangladesh is a tropical country and the average temperature of eight months in a year range from 30 ^{0}C-35 ^{0}C. Therefore, probability of getting thermotolerant *S. cerevisiae* yeast is more desirable (Mohd Azhar *et al.,* 2017) in this region.

Utilizing fermentation potential of thermotolerant *S. cerevisiae* yeast, decaying fruits can be used as an excellent feedstock for bioethanol production due to high sugar content and cost effective substrate (Shubhra *et al.,* 2014). Among the total fruit production, 25 to 60 % of fruits losses freshness due to several factors such as poor handling of the products and different diseases caused by pathogens (Janisiewicz and Korsten, 2002). Postharvest losses of fruits in Bangladesh are about 25-50 %, where it is only 5-25 % in developed countries (Kader, 1992). The utilization of these fruit wastes for bioethanol production will be one of the best and cost effective ways of saving natural resources (Vishwakarma *et al.,* 2014). Banana, Papaya, grape and apple are the most commonly consumed fruits all over the world. Due to their perishable nature, these fruits lost their freshness very quickly. Moreover, these fruits are available with the highest energy source and inverted-sugar (Jayaprakashvel *et al.,* 2014; Shubhra *et al.,* 2014), also, represent as potential energy feedstock, which may be especially suited for bioethanol production.

Bangladesh is a small country with lots of population, and the most vulnerable due to climate changes. Burning of fossil fuels worsen the situation more. Therefore, Bangladesh needs a quick and environment-friendly alternative. Biofuel can be a good option for this. Therefore, it is essential to find appropriate thermotolerant yeast isolates suitable for commercial bioethanol production. To our knowledge, this is the first study where thermotolerant yeast isolates were morphologically and molecularly screened and was determined their fermentation potentiality using decaying fruit wastes.

MATERIALS AND METHODS

Sample collection and processing: Nine different fruit samples such as guava, grape, pineapple, shaddock, lemon, banana, apple, orange and papaya were collected from different places of Mymensingh, Bangladesh during the summer season. These fruits were selected based on their year round availability, comparatively lower cost and as a constant habitat of *S. cerevisiae*. Fruits were cut into small pieces and washed in running tap water followed by distilled water. Small pieces of the fruits were kept into wrapped plastic box at 30 °C and were checked for microbial growth after one week.

Selection of *S. cerevisiae* yeast isolates based on cultural characteristics: Microbial growth observed in different fruit samples was carefully examined. *S. cerevisiae* yeast like culture were picked up and dissimilar microbial growth were discarded because of bacterial and other contamination. The selected six isolates were collected from culture of Grape, Papaya, Banana, Guava, Orange and Pineapple and hence named as Sc-Gr, Sc-Pa, Sc-B, Sc-Gu, Sc-O and Sc-Pi, respectively (Sc means *Saccharomyces cerevisiae*, Gr-Grape, Pa-Papaya, B-Banana, Gu-Guava, O-Orange and Pi-Pineapple). The isolates were sub-cultured by streak plate technique according to Jangra *et al.* (2018) on Yeast Peptone Dextrose (YEPD) agar plates and incubated at 30 ^{0}C for 24 hrs. Control strain of *S. cerevisiae* was collected from the Department of Food Technology, Bangladesh Agricultural University, Mymensingh.

Characterization of Yeast Isolates: Identification of yeasts isolates was carried out on the basis of cultural, morphological and biochemical tests. For cultural characterization different characteristics e.g; shape, color, edge, opacity, elevation, surface and consistency of isolated colonies were observed.

Morphological Characterization: In order to study the morphology of the isolates, the culture was examined microscopically according to Allen *et al.* (2006). Vegetative cells were observed after 48 hrs of incubation at 30 ^{0}C in YEPD medium. For microscopic examination, Cells were harvested by mild centrifugation (5000 rpm for 5 mins). Then cells were washed and fixed. The fixed cells (5 µl) were dropped into the well of a 10-well multi test microscope slide (76 x 26 mm with 24 x 60 mm cover slip; Matsunami glass Ind., Ltd., Japan) and was air dried. After slide preparation, staining was performed by crystal violet (Harrigan and McCane, 1982).

Biochemical characterization: Carbohydrate fermentation ability of the *S. cerevisiae* isolates was evaluated based on the utilization of carbon sources, as previously described (Matsushita *et al*., 2016; Nagodawithana *et al.*, 1976; Sanni and Lönner, 1993). A loop of yeast culture was added into the tubes of bromocresol purple carbohydrate fermentation broth (5g/l Yeast-extract, 10g/l Tryptone; 20g/l Carbohydrate i.e; Dextrose, Sucrose, Lactose and Xylose, 5g/l Sodium chloride, 1% Bromocresol purple solution and broth pH 7.5) and incubated at 30 °C for 48 hrs**.** The ability of the strains to utilize the carbon sources i.e; dextrose, lactose, sucrose and xylose and production of gas were determined in Durham's tube on carbohydrate fermentation medium.

Screening for the thermotolerance properties of *S. cerevisiae* yeast isolates: Cells from actively growing culture of different *S. cerevisiae* isolates were inoculated into conical flasks containing 50 ml of YEPD broth, incubated at water bath shaker with different temperature such as 30 ^{0}C, 37 ^{0}C, 41 ^{0}C and 45 ^{0}C for 12 hrs. According to Ginovart *et al*. (2018), 12 hrs period culture of yeast cell covers the log phase of the wild type *S. cerevisiae* and represent the active growth pattern of a specific isolate. During incubation, cell suspension was collected after 2 hrs of interval and at last (from 10 hrs) 1 hr of interval, cell growth was seized by putting the

samples on ice box. The optical density of each culture was measured by spectrophotometer (Specord UV/Visible Spectrophotometer, Analytic Jena, Germany) at 660 nm against fresh media as blank.

Molecular Screening for thermotolerant properties: Genomic DNA of *S. cerevisiae* isolates was extracted by following the protocol of Presto™ Mini gDNA Yeast kit with some modifications. In brief, cells were lysed with lysis buffer and mixed by vortexing. Then the solution was incubated for 5 mins at 95 ^{0}C in hot water bath followed by ice formation at -20 ^{0}C. The samples were then thawed at 95 ^{0}C temperature for 5 mins and were mixed by vortex. This freeze–thaw step was repeated two times followed by adding lysis buffer. Then protein removal buffer was added to the sample. After that, the solution was centrifuged at 13000 rpm for 5 mins and the supernatant was collected. 400 µl of isopropanol was added and mixed by gentle tapping. DNA was pelleted by centrifuge at 13500 rpm for 10 minutes. After careful removal of supernatant, 70% ethanol was added to wash the pellet and centrifuged at 13500 rpm for 10 mins. The ethanol was discarded, and the pellet was air dried completely. Then the DNA pellets were resuspended in an appropriate volume of DNA hydration buffer and incubated at 60 ^{0}C for 10 mins to dissolve the DNA pellet. Finally, the tubes with DNA samples were stored in freezer at -20 ^{0}C.

To perform screening of thermotolerant isolates, genomic DNA of all isolates were amplified using RSP5-C allele specific primer (Forward primer 5' GATACTGCTA CATCGAGTGG 3', Reverse primer 5' TGTTACAGAGGAATTATCTG 3') by polymerase chain reaction (PCR). Primer was designed manually from the coding region of the gene to get band of 600bp in length. RSP5-C allele was reported as one of the regulator gene for high temperature tolerance (HTG^+) phenotype by Shahsavarani *et al.* (2012). The PCR reaction was done based on protocol from Williams *et al.* (1990). 50 µl of final reaction volume was prepared and PCR conditions of an initial denaturation of 94 °C for five minutes, an annealing temperature of 60 °C, extension of 72 °C for 1 min and final extension of 72 °C for seven minutes and finally stored at 4 °C. The amplified product from each sample was separated by agarose gel electrophoresis on 1.4 % agarose gel containing ethidium bromide using 1X TBE buffer at 100V for 1.3 hrs. A 1kb DNA marker was used alongside the PCR products.

Determination of ethanol production capability of *S. cerevisiae* isolates: Ethanol production potential of *S. cerevisiae* isolates were checked in the medium by potassium dichromate oxidation method **(**Arthur and Watson, 1976**).** Then distilled product was titrated against freshly prepared ferrous ammonium sulphate solution with diphenylamine.

Utilization of *S. cerevisiae* isolates for production of bioethanol from decaying fruit wastes:

Collection, preparation and pretreatment of fruit wastes as substrate for fermentation: The fruit wastes i.e; decaying grape, apple, banana and papaya were collected from different fruit market of Mymensingh area. After cleaning, the fruits were cut and dried at 50 ºC for 5 hrs using hot air oven, and were ground to get a fine powder. The powder of fruit wastes was stored in an airtight container. Acid pre-treatment was done by hydrolysis to achieve delignification and neutralization with 1% NaOH (Braide *et al.*, 2016).

Optimization of bioethanol production using *S. cerevisiae* yeast isolates: Four *S. cerevisiae* isolates namely, Sc-Gr, Sc-Pa, Sc-B, and Sc-Pi showed good growth at 41 ºC. Among these four isolates, Sc-Gr showed the most active growth at 41ºC and was used for further study.

Log phase Culture of Sc-Gr isolate was prepared in YEPD medium and added in fermentation broth (2% peptone, 2% dextrose and 1% yeast extract) in a 500 ml conical flask. Then it was incubated in rotary shaker incubator. The slurry of the substrate was then added in the fermentation broth. The fermentation process was carried out at different ranges of parameters including temperature (30 ºC, 37 ºC, 41 ºC and 45 ºC), pH (4.5, 5.0, 6.0 and 7.0) and fermentation period (24 hrs, 48 hrs and 72 hrs). The original gravity was measured before the fermentation as the initial gravity. Data were taken and analyzed at different interval of fermentation period (24 hrs, 48 hrs and 72 hrs).

Measurement of specific gravity and bioethanol concentration: The Final gravity of wort was measured by using alcohol meter after the completion of fermentation. After measurement of the final gravity, the bioethanol concentration was calculated by using the following formula.

Here, 131= A mandated specific factor to calculate bioethanol concentration (Spedding, 2016).

$$\text{Bioethanol concentration (\%)} = \frac{(\text{Original gravity-Final gravity})}{100} \times 131$$

Statistical analysis: All analyses were carried out in three replicates by a simple one factor Analysis of Variance (ANOVA) followed by posthoc test using the Statistical Package for Social Science (SPSS) database version 16.0. *P* value <0.05 was considered as significant.

RESULTS

Collection of fruit samples for screening thermotolerant *S. cerevisiae* yeast isolates: Nine different fruit samples were kept in individual airtight box for each to grow microorganisms with the aim to screen *S. cerevisiae* yeast isolates for assessment of thermotolerance characteristics as well as ethanol production potentiality. From nine different fruits, a total of six *S. cerevisiae* yeast like colonies were selected based on their morphological characteristics for further biochemical and molecular confirmation. They were from Grape, Papaya, Banana, Guava, Orange and Pineapple and named as Sc-Gr, Sc-Pa, Sc-B, Sc-Gu, Sc-O and Sc-Pi, respectively.

Characterization of yeast isolates: White and creamy color colonies similar to *S. cerevisiae* yeast were selected. The growth of *S.cerevisiae* yeast isolates is shown at Fig. 1A. Walker & Wilson (1991) reported that culture of *S. cerevisiae* in YEPD liquid medium becomes volatile and light colored after a certain period of time. According to their study, this is because of production of gaseous and volatile compound in the culture medium. In our study, the selected isolates grown in YEPD liquid medium at 30 °C became volatile and light colored after 24 hrs of incubation (Fig. 1B).

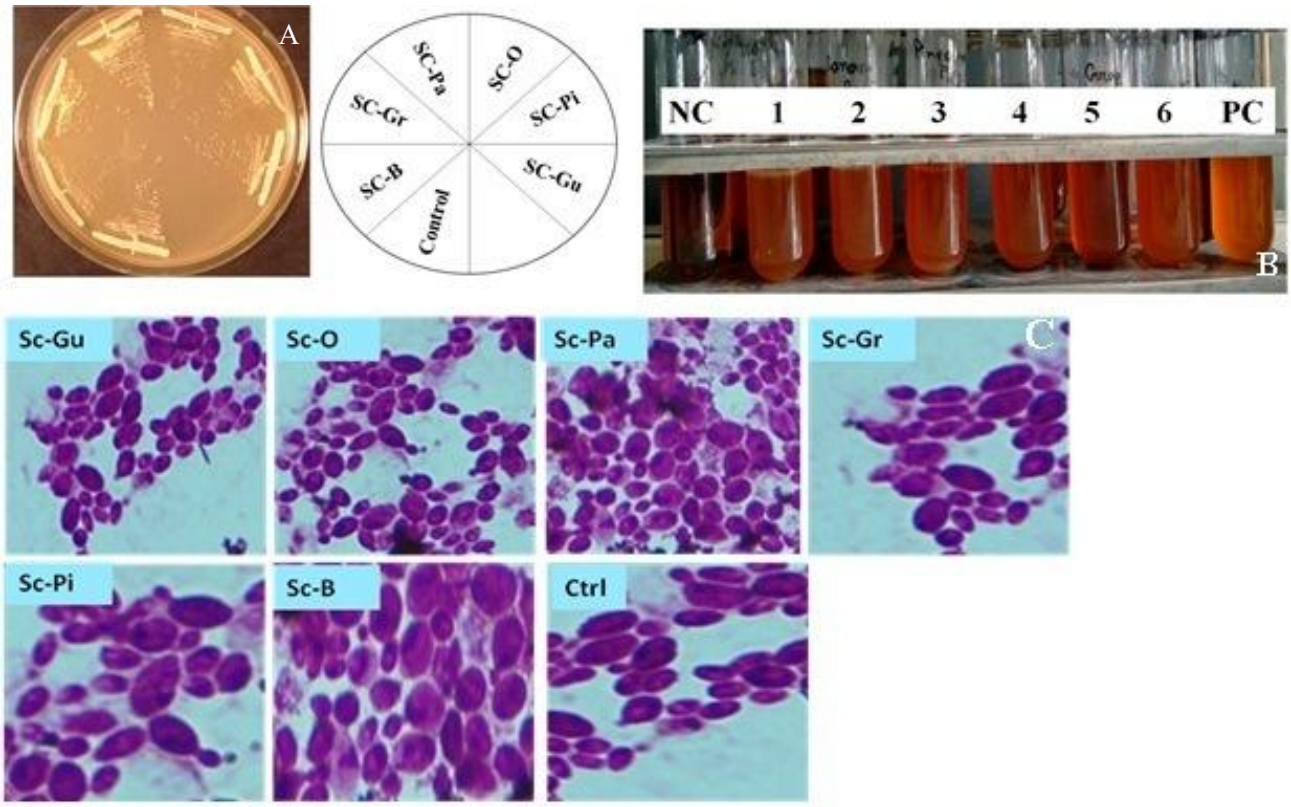

Fig. 1. Growth of yeast isolates on YEPD liquid medium. A. Streak plate culture on YEPD plate. The figure represents the uniform colonies on YEPD plates after 24 hrs of incubation at 30 °C. B. Growth of yeast isolates in YEPD broth after 24 hrs of incubation at 30 °C. Here, NC= YEPD broth without isolate, 1= Sc-B, 2= Sc-Gr, 3=Sc-Pa, 4= Sc-O, 5= Sc-Pi, 6= Sc-Gu and PC= Control strain. C. Morphological observation of cell shape of *S. cerevisiae* isolates under microscope (1000x magnification). The figure represents the morphological features of the isolates and the presence of bud. Methylene blue was used as dye.

To observe the colony characteristics, selected isolates were cultured by streak plate technique on YEPD agar plate. Colony size of the isolates was circular with creamy white color; surface edge was smooth with viscid consistency. Based on the colony characteristics (white

and creamy texture, Fig. 1A), the selected isolates were assumed as *Saccharomyces* type.

The cell morphology was studied under microscope. The cell shape was found as spherical and unicellular like *S. cerevisiae* cell**.** In this study, single and pairs of budding cells were found. Morphological characteristics of isolates are shown in Fig. 1C.

Biochemical characterization of yeast isolates: *S. cerevisiae* isolates were evaluated to check their ability to utilize different sugar as their carbon source (glucose, sucrose, lactose and xylose). In this study, the isolates utilized glucose, sucrose and xylose but could not utilize lactose. Characteristics of the isolates found in carbohydrate fermentation test were similar as the control strain. Six isolates were identified as *S. cerevisiae* based on their biochemical characteristics. Result of carbohydrate fermentation has been summarized in Table 1.

Table 1. Fermentation result of different carbohydrates by *S. cerevisiae* yeast isolates.

Carbohydrate Sources	Colour before fermentation	Carbohydrate broth after fermentation						
		Control	Sc-Gu	Sc-B	Sc-Pi	Sc-Pa	Sc-Gr	Sc-O
Glucose	Pink	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)
Sucrose		Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)
Lactose		Pink(-)	Pink (-)	Pink (-)	Pink (-)	Pink (-)	Pink (-)	Pink (-)
Xylose		Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)	Yellow (+)

Screening for thermotolerance properties of *S. cerevisiae* yeasts isolates: The growth response of isolated *S. cerevisiae* yeast cells was examined in YEPD liquid medium at four different temperatures (30 °C, 37 °C, 41°C and 45 °C) for 12 hrs. The absorbance of the culture was measured by the spectrophotometer at 660 nm based optical density (OD) at different time intervals. The growth rate was observed where the highest rate was at 30 ºC followed by growth rate at 37 ºC and the lowest was at 41 °C, respectively for all the isolates. Initially there was little growth but later there was no growth at 45 °C for all the isolates except Sc-Gr. This isolate showed outstanding growth rate at 41 ºC and little growth at even 45 ºC, too. The growth scenario of the isolated strains has been shown in Fig. 2.

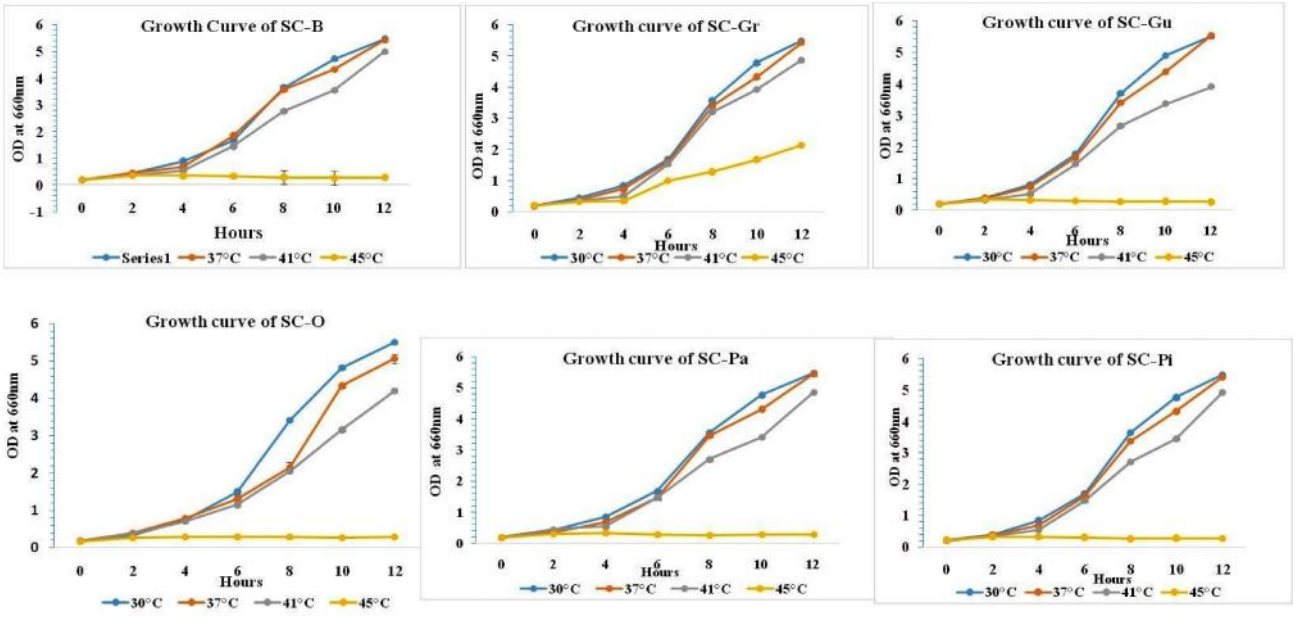

Fig.2. Effect of different temperature on the growth rate of the six different isolates of *S. cerevisiae* yeast in YEPD broth media. The X-axis represents optical density (660nm) and the Y-axis represents different time interval. The growth rate was observed for 12 hrs at 2 hrs interval.

Molecular screening for the *RSP5*-C dominant allele known to regulate thermotolerance among yeast isolates: Genomic DNA of yeast isolates were amplified by polymerase chain reaction using *RSP5-C* allele specific primer. It was reported that an Htg+ (High temperature growth) strain expressing the *RSP5-C* allele exhibits a more robust Htg+ phenotype against higher temperature (Shahsavarani *et al.,* 2012). The amplified PCR product was subjected to gel electrophoresis (Fig. 3). All the isolates showed a single band of approximately 600 bp representing the presence of dominant allele of *RSP*5 gene. A thermotolerant *S. cerevisiae* strain was used as positive control and a thermosensitive isolate was used as negative control which did not show any band. In our study, we screened six isolates containing Htg+ phenotype, recommended as thermotolerant and referred to as Htg+ phenotype.

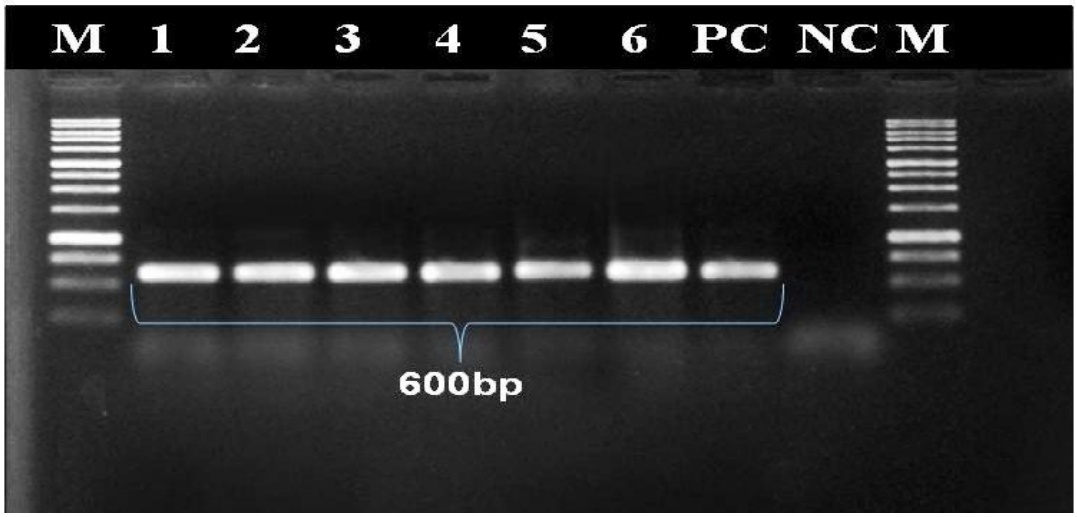

Fig.3. Gel electrophoresis result of PCR product using genomic DNA of six yeast isolates. Here, M= 1kb DNA Marker, lane 1= Sc-Gr, lane 2= Sc- Pa, lane 3 =Sc-B, lane 4 = Sc-Pi, lane 5 = Sc-O, lane 6 = Sc-Gu, PC = A thermotolerant *S. cerevisiae* strain, NC = A thermosensitive isolate of *S. cerevisiae*.

Determination of bioethanol production capability of *S. cerevisiae* isolates at shake flask: Ethanol production capability was measured by the potassium dichromate oxidation method. *S. cerevisiae* yeast isolates were cultured in YEPD media for 24 hrs and then it was distilled and checked to observe the potential for bioethanol production. Diphenylamine was used as an indicator. The appearance of green colour at the end point indicated the presence of ethanol produced (Fig.4).

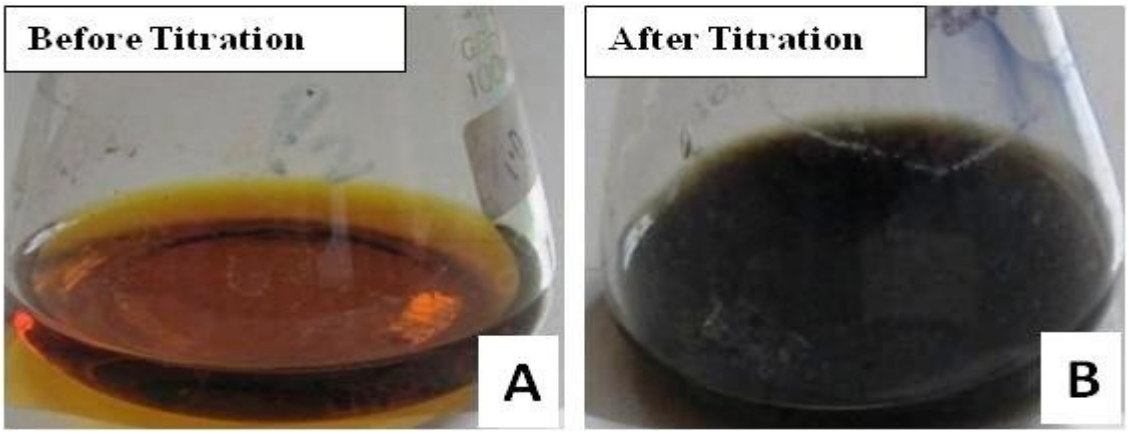

Fig.4. Determination of ethanol production potential of Sc-Gr isolate in YEPD liquid media at 41 °C. Here, Fig. 4A shows the distilled culture media before titration and Fig. 4B shows the distilled culture media after titration. The appearance of green color after titration indicates the presence of ethanol in Fig. 4B. All the isolates were tested and found potential. Here we present only Sc-Gr isolates as representative of the isolates.

Selection of active *S. cerevisiae* isolate for bioethanol production: Among six *S. cerevisiae* yeast isolated in this study, four isolates namely, Sc-Gr, Sc-Pa, Sc-B and Sc-Pi showed comparatively better growth at higher temperature as 41 ºC (Fig. 5A). Among these four isolates, Sc-Gr showed the most active growth at 41 ºC (Fig. 5B) and was used to produce bioethanol from decaying fruits.

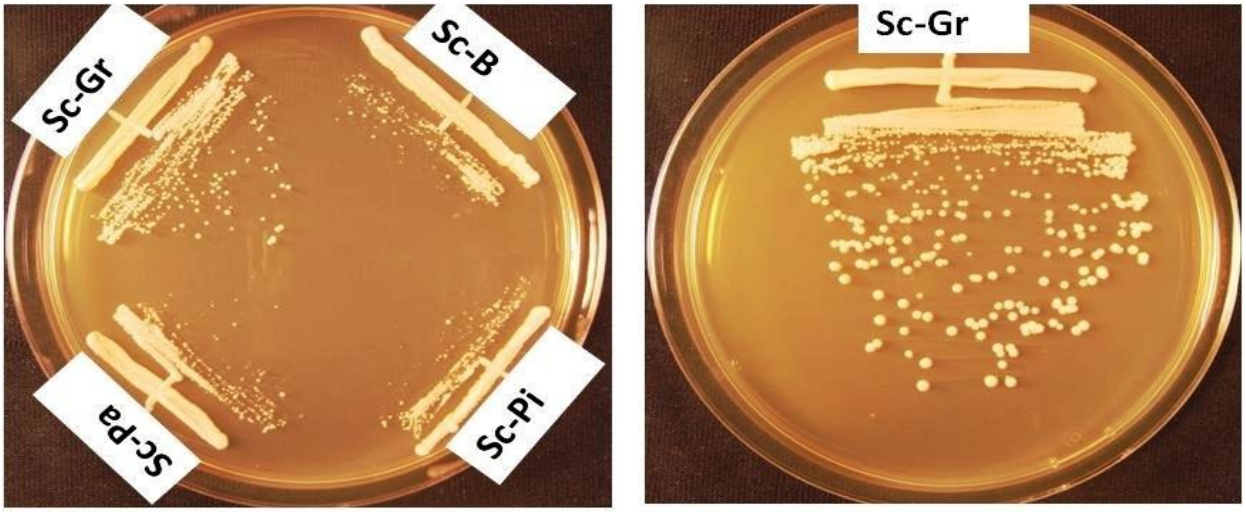

Fig.5. A. Growth of four yeast isolates on YEPD plate at 41 ºC B. Growth of Sc-Gr on YEPD plate at 41 ºC. Photos were taken after 24 hrs of incubation.

Optimization of different parameters for bioethanol production: In the present study, the effect of temperature, pH, fermentation period and substrate on bioethanol production were analysed from four decaying fruit samples, i.e; apple, grape, papaya and banana.

Effect of different temperature on bioethanol production: Optimization of the effective fermentation temperature is very crucial for bioethanol production. The bioethanol concentration was measured from fermentation of four fruit wastes at different temperatures (30 ºC, 37 ºC, 41 ºC and 45 ºC) where pH of the fermentation broth was 5.0 and data were collected after 72 hrs of fermentation period. Maximum bioethanol concentration (7.89 %) was obtained from decaying grapes at 37 ^{0}C, and the minimum amount of bioethanol concentration (2.37 %) was obtained from decaying papaya while the temperature was 30 ºC. Grape and apple wastes produced a significantly higher amount of bioethanol than banana and papaya wastes. The isolate Sc-Gr produced a significant amount of bioethanol even at 41 ºC (Table 2).

Effect of different pH on bioethanol concentration: Bioethanol concentration using decaying fruits samples were measured from fermentation broth having different pH such as 4.5, 5.0, 6.0 and 7.0 (Table 3). Data revealed that the maximum amount of bioethanol concentration (7.78%) was obtained from grape waste while pH of the fermentation broth was 5.0 followed by apple waste (7.61%) at pH 6.0. The lowest bioethanol concentration (2.76%) was obtained from papaya waste at pH 7.0 followed by 2.83% from banana waste at pH 7.0.

Table 2. Effect of different temperature on bioethanol concentration (%). pH of the fermentation broth was 5.0 and data were collected after 72 hrs of fermentation period

Fruit wastes	Temperature	Bioethanol concentration (%Mean ±SD)
Apple	30 ^{0}C	7.74 ± 1.15 ^{a}
	37 ^{0}C	7.01 ± 0.50^{bc}
	41 ^{0}C	6.53 ± 0.70 ^{cd}
	45 ^{0}C	6.02 ± 0.80^{d}
Grape	30 ^{0}C	6.09 ± 0.60 ^{d}
	37 ^{0}C	7.89 ± 1.50 ^{a}
	41 ^{0}C	7.31 ± 1.75 ^{ab}
	45 ^{0}C	6.80 ± 1.15 ^{bc}
Papaya	30 ^{0}C	3.85 ± 0.75 ^{ef}
	37 ^{0}C	6.02 ± 0.95 ^{d}
	41 ^{0}C	2.75 ± 0.45 ^{h}
	45 ^{0}C	2.37 ± 0.35 ^{h}
Banana	30 ^{0}C	4.37 ± 0.65 ^{e}
	37 ^{0}C	3.52 ± 0.55 ^{fg}
	41 ^{0}C	2.90 ± 0.49 ^{gh}
	45 ^{0}C	2.38 ± 0.41 ^{h}
Level of significance		**
$LSD_{(0.05)}$		0.675
CV (%)		7.67

Note: Column with the same letters does not differ significantly, whereas column with different letters differ significantly at 5% probability level.

Table 3. Effect of different pH on bioethanol concentration (%). Temperature was adjusted at 37 ºC and data were collected after 72 hrs of fermentation period

Fruit wastes	pH	Bioethanol concentration (%Mean ±SD)
Apple	4.5	5.97 ± 1.11 [d]
	5.0	7.24 ± 1.41 [b]
	6.0	7.61 ± 1.50 [ab]
	7.0	6.54 ± 1.21 [c]
Grape	4.5	7.25 ± 1.45 [b]
	5.0	7.78 ± 1.77 [a]
	6.0	6.63 ± 1.35 [c]
	7.0	5.47 ± 1.10 [e]
Papaya	4.5	3.65 ± 1.0 [gh]
	5.0	3.93 ± 1.11 [fg]
	6.0	3.28 ± 1.0 [h]
	7.0	2.76 ± 0.91 [i]
Banana	4.5	3.91 ± 1.00 [fg]
	5.0	4.28 ± 1.20 [f]
	6.0	3.76 ± 1.15 [g]
	7.0	2.83 ± 0.90 [i]
Level of significance		**
$LSD_{(0.05)}$		0.402
CV (%)		4.64

Note: Column with the same letters does not differ significantly, whereas column with different letters differ significantly at a 5% probability level.

Effect of different fermentation periods on bioethanol concentration: Bioethanol concentration obtained from four decaying fruit samples at different fermentation period such as 24 hrs, 48 hrs and 72 hrs are shown in Table 4. Here, the temperature was set at 37 °C and pH of the fermentation broth was 5.0. The significantly highest bioethanol concentration (7.92 %) was obtained from grape wastes after 48 hrs of fermentation period followed by 7.60 % from apple after 72 hrs of fermentation period. On the other hand, the lowest bioethanol concentration (2.45%) was obtained from banana waste after 24 hrs of fermentation period followed by 2.86% from papaya after 72 hrs of fermentation period. These results indicate that fermentation period plays an important role in bioethanol production.

Table 4. Effect of fermentation period on bioethanol concentration from different fruit wastes. Fermentation temperature was 37 ºC and pH of the fermentation broth was 5.0.

Fruit wastes	Fermentation period (hrs)	Bioethanol concentration (%Mean ±SD)
Apple	24	6.12 ± 1.10 [c]
	48	6.88 ± 1.40 [b]
	72	7.60 ± 1.80 [a]
Grape	24	6.34 ± 1.20 [c]
	48	7.92 ± 1.70 [a]
	72	6.19 ± 1.40 [c]
Papaya	24	2.97 ± 1.0 [f]
	48	3.82 ± 1.40 [e]
	72	2.86 ± 1.10 [f]
Banana	24	2.45 ± 1.20 [g]
	48	3.57 ± 1.25 [e]
	72	4.35 ± 1.30 [d]
Level of significance		**
$LSD_{(0.05)}$		0.404
CV (%)		4.69

Note: Column with the same letters does not differ significantly, whereas column with different letters differ significantly at a 5% probability level.

Effect of different substrate on bioethanol concentration: Bioethanol concentration was measured from fermentation of different fruit wastes as samples and data were compared. The significantly highest amount of bioethanol concentration was obtained from grape waste (7.83% ± 0.2) followed by apple waste (7.56% ± 0.17). However, the lowest amount of bioethanol (3.91% ± 0.11) was obtained from papaya waste followed by bioethanol concentration (4.3% ± 0.27) obtained from banana waste (Fig.6).

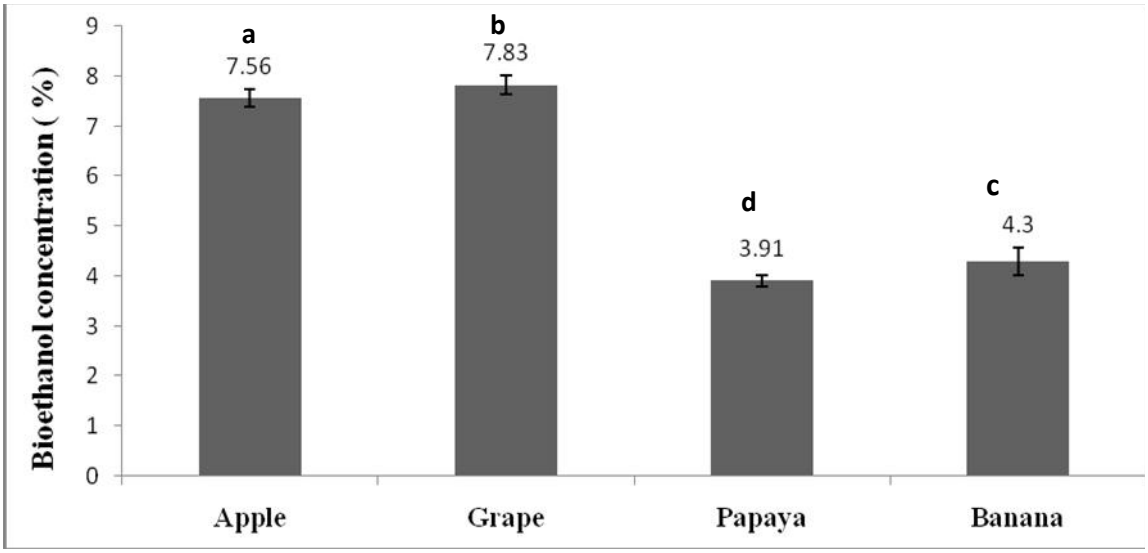

Fig.6. Effect of different fruit wastes as substrate on bioethanol concentration (%).Temperature was 37 ºC, pH of the fermentation broth was 5.0 and fermentation period was 72 hrs.

DISCUSSION

The world's dependency on fossil fuels has caused unfavourable effects on health, environment and economy. On the other hand, bioethanol has opened a new window of blend or fossil petrol substitute to overcome these challenges, simultaneously promoting sustainability and independence from fossil fuel (Aditiya *et al.,* 2016). Current strategies to produce fuel ethanol employ simultaneous saccharification and fermentation (SSF) that has been applied in ethanol fermentation from starch-based feedstocks. The main drawback of SSF is its compromised optimal temperature between hydrolysis and fermentation steps, as the lower temperature for hydrolysis is considered high for the fermenting cells, consequently influencing the overall activity of hydrolysis and fermentation (Kassim *et al.,* 2022). Therefore, temperature optimization is an urgent need for enzymatic hydrolysis and fermentation. *S. cerevisiae* is widely used in the fermentation industries for its multiple potentialities. *S. cerevisiae* shows good fermentative capabilities at low temperatures; however, at high temperatures, numbers of viable cells decrease (Torija *et al.,* 2003). Fortunately, thermotolerant yeast strains can tolerate high temperatures compatible with optimal cellulase and hemicellulase activities, producing a significantly high ethanol yield and reducing production costs (Zhao and Bai, 2009).

In this study, we have screened six thermotolerant *S. cerevisiae* yeast isolates from common fruits of Bangladesh during the summer season. Cultural, morphological and biochemical characteristics were analyzed and confirmed as *S. cerevisiae*. The findings were in line with Guimarães *et al.* (2006). Molecular confirmation was done using *RSP5-C* allele-specific primer, which is already reported for its involvement in high-temperature tolerance phenotype. In a previous study, *HTG* (High-Temperature Growth) genes were found responsible for Htg+ phenotype in the thermotolerant *S. cerevisiae* strain (Benjaphokee *et al.,* 2011). After that, a follow-up study by Shahsavarani *et al.* (2012) revealed that the *RSP5-C*, an allele of the gene *RSP5* encoding a ubiquitin ligase, was correlated with the thermotolerant phenotype of *S. cerevisiae*, and the underlying mechanism might be the activation of the enzyme by the increased transcription of this gene under thermal stress conditions. As Bangladesh is a tropical country, it was assumed that thermotolerant *S. cerevisiae* isolates could be screened from different fruit samples during summer season**.** In this study, the Sc-Gr isolate showed outstanding growth rate at 41 ºC and little growth at even 45 ºC revealing its high temperature tolerance with Htg+ phenotype. A recent study in Bangladesh by Talukder *et al.* (2016) also found *Pichia kudriavzevii* as potential thermotolerant yeast strain after screening thermotolerant microorganisms from nine fermented natural sources like boiled potatoes, municipal solid and liquid wastes and sugarcane juice. This strain can tolerate up to 42 °C temperature while producing higher bioethanol. There are many reports on screening of thermotolerant *S. cerevisiae* isolates from other tropical countries. Jayamma *et al.* (2017) in India isolated and identified thermotolerant yeast from fruits wastes sapota, papaya, mango, pineapple, banana, grapes and orange. They found 20 isolates capable of growing at 45 ºC and 70 strains at 40 °C**.** Another study screened three strains of *S. cerevisiae,* two widely used strains from the Brazilian ethanol industry (CAT-1 and PE-2), and another can grow at 42 °C (LBM-1) for cellulosic ethanol production, which are rare in yeasts belonging to the *S.*

cerevisiae species and concluded its potential in industrial processes (Costa *et al*., 2014).

Suitable temperature in fermentation process is a vital factor (Rivere *et al.,* 2006) for bioethanol production. The present study observed the significantly highest bioethanol concentration (7.92 %) from grape waste at 37 ^{0}C at pH 5.0 after a 48 hrs fermentation period using Sc-Gr yeast isolate. Our result is similar with the findings of Raikar (2012), where they observed maximum bioethanol concentration from grape after 48 hrs of fermentation at temperature 35 °C having broth pH 5.0. A similar study by Mohamed *et al.* (2013) observed the highest ethanol concentration of 11.72% at 35 °C *Kluyveromyces* sp. after 96 hrs of fermentation period. The temperature has a direct effect on the biochemical reactions and metabolism of yeasts because the enzyme activities are suppressed at low temperature and the higher temperature affects the alcohol production because of cell death (Gao and Fleet, 1988).

Considering the pH of the fermentation broth, a slightly acidic medium (pH 5) was proven to be the most appropriate pH for bioethanol production. Narendranath and Power (2005) found that the optimum pH for yeast growth and ethanol production by *S. cerevisiae* was pH 4.9, whereas Pramanik (2003) reported that the maximum ethanol production by *S. cerevisiae* was achieved for pH 4.25–5.0. In a study by Pramanik (2003), it was argued that the activity of the yeast species at pH 3.75 was decreased and this was attributed to the inability of the activation of the fermentation enzymes at such a low pH. On the other hand, at the higher pH values tested, the low ethanol production and sugar conversion values could be due to the formation of undesired products like glycerol and organic acids (Zohri and Etnan, 2000).

The fermentation time seems quite an important factor since it affects the productivity of the process (Hashem *et al*., 2019). Conversion of sugar to ethanol takes significant duration for fermentation, and this period is different from one substrate to another (Neelakandan and Usharani, 2009). In this study, the significantly highest bioethanol concentration (7.92 %) was obtained from grape wastes after 48 hrs of fermentation period followed by 7.60 % from apple after 72 hrs of fermentation period. Results obtained from this study are almost similar with the findings of Neelakandan and Usharani (2009). These scientists obtained maximum yield of ethanol (7.62%) from Grape at pH 6.0, temperature 32.5 °C after 24 hrs of fermentation period. Longer fermentation times are reported for the achievement of maximum bioconversion and ethanol production in other research findings (Limtong *et al*., 2007, Zohri and Etnan, 2000, Sarower *et al*., 2020). Efficient management of the operational parameters crucial for fermentation process is expected to ensure the higher bioethanol yield.

Conclusion: Bioethanol production from renewable waste material has tremendous economic and ecological significance. The direct microbial conversion of starch and other hemicelluloses from fruit waste to ethanol is of great economic significance. From this study, we successfully screened thermotolerant *S. cerevisiae* isolates from the available fruits of Bangladesh. The isolate Sc-Gr of this study showed thermotolerance and produced the highest amount of bioethanol from different fruit wastes. Therefore, further research on this isolate focusing on the overexpression of *RSP5*, a gene responsible for thermotolerance in *S. cerevisiae* yeast under ethanol fermentation conditions is necessary to confirm the possibility of exploiting this technique commercially for industrial bioethanol production. Proper and efficient utilization of fruit wastes for bioethanol production will be an environment-friendly and sustainable production source of renewable energy in the future.

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

Aditiya, H.B., T.M.I. Mahlia, W.T. Chong, Hadi Nur, and A.H. Sebayang (2016). Second generation bioethanol production: A critical review, Renew. Sust. Energ. Rev. 66: 631-653. DOI: 10.1016/j.rser.2016.07.015

Alfenore, S., C. Molina-Jouve, S.E. Guillouet, J.L. Uribelarrea, G. Goma and L. Benbadis (2002). Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process. Appl. Microbiol. Biotechnol. 60: 67–72. DOI: 10.1007/s00253-002-1092-7

Allen, C., S. Büttner, A.D. Aragon, J.A. Thomas, O. Meirelles, J.E. Jaetao, D. Benn, S.W. Ruby, M. Veenhuis, F. Madeo and M. Werner-Washburne (2006). Isolation of quiescent and nonquiescent cells from yeast stationary-phase cultures. J. Cell. Biol. 174(1): 89–100. DOI: 10.1083/jcb.200604072

Arthur, H. and K. Watson (1976). Thermal adaptation in yeast: growth temperatures, membrane lipid, and cytochrome composition of psychrophilic, mesophilic, and thermophilic yeasts. J. Bacteriol. 128: 56-68. doi: 10.1128/jb.128.1.56-68.1976

Banat, I. M., P. Nigam, and R. Marchant (1992). Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. World J. Microbiol. Biotechnol. 8: 259–263. DOI: 10.1007/BF01201874

Benjaphokee, S., D. Hasegawa, D. Yokota, T. Asvarak, C. Auesukaree, M. Sugiyama, Y. Kaneko, Y.C. Boonchird and S. Harashima (2012). Highly efficient bioethanol production by a Saccharomyces cerevisiae strain with multiple stress tolerance to high temperature, acid and ethanol. New Biotechnol. 29: 379-386. DOI: 10.1016/j.nbt.2011.07.002

Braide, W., I.A. Kanu, U.S. Oranusi and S.A. Adeleye (2016). Production of bioethanol from agricultural waste. J. Fundam. Appl. Sci. 8: 372-386. DOI:10.4314/jfas.v8i2.14

Cho, C., S.Y. Choi, Z.W. Luo and S.Y. Lee (2015). Recent advances in microbial production of fuels and chemicals using tools and strategies of systems metabolic engineering. Biotechnol. adv. 33: 1455-1466. DOI: 10.1016/j.biotechadv.2014.11.006

Costa, D.A., de Souza, C.J.A. Costa, P.S. (2014). Physiological characterization of thermotolerant yeast for cellulosic ethanol production. Appl. Microbiol. Biotechnol. 98: 3829–3840. https://doi.org/10.1007/s00253-014-5580-3

Cot, M., M.O. Loret, J. François and L. Benbadis (2007). Physiological behaviour of *Saccharomyces cerevisiae* in aerated fed-batch fermentation for high level production of bioethanol. FEMS Yeast Res. 7: 22-32. DOI: 10.1111/j.1567-1364.2006.00152.x

Gao, C. and G.H. Fleet (1988).The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. J. Appl. Bacteriol. 65: 405–409. https://doi.org/10.1111/j.1365-2672.1988.tb01909.x

García, A., C. Cara, M. Moya, J. Rapado, J. Puls, E. Castro and C. Martín (2014). Dilute sulphuric acid pretreatment and enzymatic hydrolysis of *Jatropha curcas* fruit shells for ethanol production. Ind. Crops. Prod. 53:148-153. https://doi.org/10.1016/j.indcrop.2013.12.029

Ginovart, M., R. Carbó, M. Blanco and X. Portell (2018). Digital image analysis of yeast single cells growing in two different oxygen concentrations to analyze the population growth and to assist individual-based modeling. Front. Microbiol. 8: 26-28. https://doi.org/10.3389/fmicb.2017.02628

Guimarães, T. M., D.G. Moriel, I.P. Machado, C.M.T.F. Picheth and T.M.B. Bonfim (2006). Isolation and characterization of *Saccharomyces cerevisiae* strains of winery interest. Braz. J. Pharm. Sci. 42: 119–126. https://doi.org/10.1590/S1516-93322006000100013

Hansen, A.C., Q. Zhang and P.W. Lyne (2005). Ethanol–diesel fuel blends—a review. Bioresour. Technol. 96:277-285. DOI: 10.1016/j.biortech.2004.04.007

Harrigan, W. F. and M. E. McCance (1976). Laboratory methods in food and dairy microbiology. Academic Press Inc. Limited, London. DOI: 10.4236/ce.2016.714189

Hashem, M., T.Y.A. Asseri, S.A. Alamri and S.A. Alrumman (2019). Feasibility and sustainability of bioethanol production from starchy restaurants' bio-wastes by new yeast strains. Waste Biomass Valor. 10: 1617–1626. https://doi.org/10.1007/s12649-017-0184-7

Hossain, M.S., M.A. Habib, S. Saha, F. Akhtar, S. Yasmin and F. Khatun (2020). Production of Bioethanol from Agricultural Wastes Utilising Fermentation Potential of Saccharomyces cerevisiae. J. Bangladesh Agril. Univ. 18(4): 975–981. https://doi.org/10.5455/JBAU.8647

Jangra, M.R., S. Jangra, R. Kumar and K.S. Nehra (2018). A Study on Yeast Micro Flora Isolated From Common Indian Fruits and Cheese. Int. j. pure. appl. biosci. 6: 828-839. DOI:10.18782/2320-7051.6095

Janisiewicz, W.J. and L. Korsten (2002). Biological control of postharvest diseases of fruits. Annu. Rev.Phytopathol. 40: 411-441. DOI:10.1146/annurev.phyto.

Jayamma, P., G.G. Shirnalli, D.G. Goudar and S.N. Olekar (2022). Isolation and identification of thermotolerant yeast isolates from different fruit wastes. The Pharma Innov. J. 11(1): 855-859. http://www.thepharmajournal.com/

Jayaprakashvel, M., S. Akila, M. Venkatramani, S. Vinothini, S.J. Bhagat and A.J. Hussain (2014). Production of bioethanol from papaya and pineapple wastes using marine associated microorganisms. Biosci. Biotechnol. Res. Asia. 11: 193-199. **DOI :** http://dx.doi.org/10.13005/bbra/1410

Kader, A.A. (1992). Postharvest technology of horticultural crops, second ed. Publication No. 3311. Division of Agricultural and Natural Resource, University of California.

Kassim, M.A., Meng, T.K., Kamaludin, R., Hussain, A.H. and N.A. Bukhari (2022). Bioprocessing of sustainable renewable biomass for bioethanol production. In *Value-Chain of Biofuels* (pp. 195-234). Elsevier.

Limtong, S., C. Sringiew and W. Yongmanitchai (2007). Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. Bioresour. Technol. 98: 3367–3374. DOI: 10.1016/j.biortech.2006.10.044

Matsushita, K., Y. Azuma, T. Kosaka, T. Yakushi, H. Hoshida, R. Akada, and M. Yamada (2016).Genomic analyses of thermotolerant microorganisms used for high temperature fermentations. Biosci. Biotechnol. Biochem. 80: 655-668. DOI: 10.1080/09168451.2015.1104235

Mohamed, H., N.A.Z. Abdel and M.A. Maysa (2013). Optimization of the fermentation conditions for ethanol production by new thermotolerant yeast strains of *Kluyveromyces* sp. African Journal of Microbiology Research.7: 4550-4561.

Mohd Azhar, S.H.R. Abdulla, S.A. Jambo, H. Marbawi, J.A. Gansau, A.A. Mohd Faik, and K.F. Rodrigues (2017). Yeasts in sustainable bioethanol production: A review. Biochem. Biophys. Rep. 10: 52–61. DOI: 10.1016/j.bbrep.2017.03.003

Mussatto, S.I., G. Dragone, P.M. Guimarães, J.P.A. Silva, L.M. Carneiro, I.C. Roberto, A. Vicente, L. Domingues and J.A. Teixeira (2010). Technological trends, global market and challenges of bio-ethanol production. Biotechnol. adv. 28: 817-830. DOI: 10.1016/j.biotechadv.2010.07.001

Nagodawithana, T.W., C. Castellano and K.H. Steinkraus (1974). Effect of dissolved oxygen, temperature, initial cell count, and sugar concentration on the viability of *Saccharomyces cerevisiae* in rapid fermentations. Appl. Microbiol. 28: 383-391. doi: 10.1128/am.28.3.383-391.1974

Narendranath, N.V. and R. Power (2005). Relationship between pH and medium dissolved solids in terms of growth and metabolism of lactobacilli and *Saccharomyces cerevisiae* during ethanol production. Appl. Environ. Microbiol.71: 2239–2243. DOI: 10.1128/AEM.71.5.2239-2243.2005

Neelakandan, T. and G. Usharani (2009). Optimization and production of bioethanol from cashew apple juice using immobilized yeast cells by *Saccharomyces cerevisiae*. Am.-Eurasian J.Sci.Res. 4:85-88. http://www.idosi.org/aejsr/4(2)09/7.pdf

Nonklang, S., B.M. Abdel-Banat, K. Cha-Aim, N. Moonjai, H. Hoshida, S. Limtong, M. Yamada, and R. Akada (2008). High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl. Environ. Microbiol. 74:7514-7521. DOI: 10.1128/AEM.01854-08

Olofsson, K., B. Palmqvist, and G. Lidén (2010). Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnol. Biofuels. 3: 1-9. https://doi.org/10.1186/1754-6834-3-17

Pramanik, K. (2003). Parametric studies on batch alcohol fermentation using *Saccharomyces* yeast extracted from toddy. J. Chin. Inst.Chem. Eng. 34: 487–492. https://doi.org/10.32628/IJSRST

Pretorius, I.S., M. Du Toit and P. Van Rensburg (2003). Designer yeasts for the fermentation industry of the 21st century. Food Technol. Biotechnol. 41: 3-10. https://hrcak.srce.hr/110923

Raikar, R.V. (2012). Enhanced production of Ethanol from grape waste. Int. J. Environ. Sci. 3: 776-783.

Rivera, E.C., A.C. Costa, D.I.P. Atala, F. Maugeri, M.R.W. Maciel and R.M. Filho (2006). Evaluation of optimization techniques for parameter estimation: Application to ethanol fermentation considering the effect of temperature. Process Biochem. 41: 1682–1687. https://doi.org/10.1016/j.procbio.2006.02.009

Sanni, A.I. and C. Lönner (1993). Identification of yeasts isolated from Nigerian traditional alcoholic beverages. Food Microbiol. 10: 517–523. https://doi.org/10.1006/fmic.1993.1057

Shahsavarani, H., M. Sugiyama, Y. Kaneko, B. Chuenchit and S. Harashima (2012). Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of *RSP5* ubiquitin ligase. Biotechnol. adv. 30: 1289-1300. DOI: 10.1016/j.biotechadv.2011.09.002

Shubhra, T., S.K. Jadhav, S. Mayuri, and K.L. Tiwari (2014). Fermentation of waste fruits for bioethanol production. Asian J. Biol. Sci. 7: 30-34. https://scialert.net/abstract/?doi=ajbs.2014.30.34

Spedding G, H. Bamforth and W. Charles (2016). Alcohol and its Measurement. Brewing Materials and Processes. Academic Press, San Diego, pp. 123-149.

Suutari, M., K. Liukkonen and S. Laakso (1990). Temperature adaptation in yeasts: The role of fatty acids. J. Gen.Microbiol. 136: 1469–1474. DOI: 10.1099/00221287-136-8-1469

Talukder, A.A., Easmin, F., Mahmud, S. A. and M. Yamada (2016). Thermotolerant yeasts capable of producing bioethanol: isolation from natural fermented sources, identification and characterization. Biotechnology &

Biotechnological Equipment. 30: 1106-1114. https://doi.org/10.1080/13102818.2016.1228477

Torija, M.J., G. Beltran, M. Novo, M. Poblet, J.M. Guillamón, A. Mas and N. Rozès (2003). Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. Int. J. Food Microbiol. 85: 127–136. DOI: 10.1016/s0168-1605(02)00506-8

Torija, M.J., Rozes, N., Poblet, M., Guillamón, J.M., and A. Mas (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. International journal of food microbiology. 80: 47-53. DOI: 10.1016/s0168-1605(02)00144-7

Vishwakarma, H.S., A. Kumar, J. Singh, S. Dwivedi and M. Kumar (2014). Production of Ethanol from Fruit Wastes by using *Saccharomyces cerevisiae*. Int. J. Renew. Energy Technol. Res. 3: 1-5. http://ijretr.org

Walker, L.P. and D.B. Wilson (1991). Enzymatic hydrolysis of cellulose: An overview. Bioresour. Technol. 36: 3–14. https://doi.org/10.1016/0960-8524(91)90095-2

Williams, J.G.K., A.R. Kubelic, K.J. Livak, J.A. Rafalski and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535. doi: 10.1093/nar/18.22.6531

Zhao, X.Q. and F.W. Bai (2009). Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J. Biotechnol. 144: 23-30. DOI: 10.1016/j.jbiotec.2009.05.001

Zohri, A.N.A. and M.M. Etnan (2000). Ethanol production from dates in Saudi Arabia on industrial scale. Mycobiology, 28: 76–81. https://doi.org/10.1080/12298093.2000.12015727.