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Isolation and characterization of *Candida tropicalis* from mixed fruit wastes for bio-ethanol production

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Abstract— *The bioethanol production from the biomass is gaining popularity now-a-days all over the world. The bio-ethanol is produced by the process of fermentation of starch, sugars of fruit wastes. Bioethanol can be utilized as bio-fuel for the transport purpose, sanitizer production. Compared to any other sources for bio ethanol production, mixed fruit wastes were cheaper. The present study focussed on utilization of the fruit wastes which are discarded from the local fruit shops and fresh juice shops for the bioethanol production. Mixed fruit wastes were collected and used as the raw material for the bioethanol production takes place via., Candida tropicalis. Yeast is the common species for bio-ethanol, alcohol, biofuel, wine and cider fermentation and conversion of biomass into ethanol. The mixed fruit waste can be converted into biofuel which can acts as an alternative source of bio-ethanol.*

Keywords— ***Bioethanol, Yeast isolates, mixed fruit wastes, Candida tropicalis***

I. INTRODUCTION

Because it is environmentally beneficial, bioethanol has attracted a lot of attention as a substitute energy source. The current industry and academics' top priority is to develop cost-effective technology for bioethanol production. Cost-effective technology is influenced by the following factors: quick and efficient conversion of carbohydrates to ethanol.

This study was conducted to manufacture bioethanol using economical and environmentally acceptable methods for use as a sanitizer in the current COVID-19 pandemic scenario. Also, bioethanol is a suitable commercial product because it is a petroleum-free substance that may be easily produced from agricultural feedstock or fruit and juice waste. In the study, KMnO4 (5%) was derived from the waste of various fruits and fruit juices (Chitranshi and Kapoor, 2021).

Based on phylogenetic research and nucleotide homology, the sample that was initially identified as yeast was really Candida tropicalis, which demonstrated a high degree of resemblance during the fermentation process. Desirable fermentation characteristics were found in Candida tropicalis strains that were isolated from mixed fruit wastes. Without using the saccharification phase, bioethanol was produced using *C. tropicalis*, both free and immobilized in calcium alginate. There are few reports of *C. tropicalis* strains being employed as ethanol-producing ones, despite the fact that C. tropicalis has the potential to be a valuable agent in the commercial production of bioethanol. This research study has been reported as the pioneer work in bioethanol production by *Candida tropicalis* in mixed fruit wastes (Hermansyah *et al.*, 2015). All the selected over ripened fruit wastes were analysed for variations in parameters like stress, thermal, osmatic, exogenous ethanol tolerance, invertase activity, ADH

activity, including levels of inoculum, pH and temperature concentration in complete fermentation of bioethanol production.

II. METHODS AND MATERIALS

Sample collection and isolation of yeast isolates

The mixed fruit wastes were collected from shops from different locations of Chidambaram town. Collected samples were surface sterilized by using 5% Potassium permanganate ($KMnO_4$) and rinsed with sterile water thoroughly. Individual samples were pulverized in a mixer and collected in 1 litre conical flask, sealed with a cotton stopper and covered with aluminium foil for two weeks to ferment naturally. Malt Yeast Agar (MYA) was the media that was made with 3.0g of malt extract, 3.0g of yeast extract, 5.0g of peptone, 10.0g of glucose (dextrose), 20.0g of agar, and a final pH of 6.2±0.2 at 25°C. Following the completion of the fermentation process, the cultures were separated using the pour plate method and incubated for 48 hours at 25–30 degrees Celsius.

Designation of yeast isolates isolated from mixed fruit wastes

The mixed fruit wastes were isolated from various fruit juice shops of Chidambaram town, were designated as Yi-1, Yi-2, Yi-3, Yi-4, Yi-5 sequentially.

Morphological characterization of yeast isolates

The yeast isolates were recognized depending on their Visual traits and Biochemical assays. After 48 hours of growth, the morphological parameters were observed for each yeast colony comprised colony appearance, texture, margin, shape, size, form, height, extremity, visual properties, regularity, colony facet, and tint, multilateral budding, sediment, pseudohypha, pellicle and ascospore formation.

Biochemical characterization of yeast isolates

Urease test

A tiny amount of 24-hour-old culture was transferred, and after 48 hours at 30 degrees Celsius, it was inoculated in urease agar; the appearance of a reddish-pink color indicated a successful outcome. (Tambuwa *et al.*, 2018).

Carbohydrate fermentation and assimilation test

The ability of a culture to ferment sugars with the production of gas and acid was tested using sugar. A peptone water medium containing 1% fermentable sugar and 0.1 % phenol red was used to make sugar indicator broth. Ten milliliters of sugar broth were added to each test tube, and the Durham tube—which would catch any gas that could form—was gently inverted. A loopful of the 48-hour-old yeast cultures was autoclaved and used to inoculate the test tubes. The tubes were then cultured for two to seven days at 36°C, with daily checks for the production of gas and acid. The generation of acid is shown by the yellow coloration, and the displacement of the medium in the Durham tube indicates the production of gas. All the yeast spp. were tested for the utilization and assimilation of carbon sources (A Reddick, 1975).

Efficiency tests of yeast isolates

All ten isolates of yeast obtained from the mixed fruit waste samples were screened for their bioethanol production.

Thermal tolerance

Five milliliters of phosphate buffer were added to a test tube containing one milliliter of each of the chosen yeast isolates. The suspension was kept in a water bath set at different temperatures *viz*, 35º, 40º, 45º, 50º, 55º and 60ºC. The test tubes were removed and rapidly cooled after 20 minutes of exposure. Then, 1 mL of sample from each tube was serially diluted and plated on MYA medium, to determine the viability of yeast cells respectively.

Osmotic tolerance

At various temperatures, one mL of the selected yeast cells isolates was examined. Each thermally equilibrated cell pellet was then immediately combined with glycerol solution at the same temperature. Cell viability was tested in MYA medium, and the cells were slowly reheated to ambient temperature (25ºC) for 15 minutes and rehydrated by adding water (25ºC). The thermocouple was used to measure the temperature of the glycerol solution and cell pellets.

Exogenous ethanol tolerance

After being cultivated on MYA agar medium with varying ethanol concentrations of 5, 10, 15, 20, and 25% (v/v), six distinct chosen yeast species were inoculated with an initial cell concentration of 1×106 cells/ml. The cultures were kept at 35°C for 48 hours of incubation. The use of a hemocytometer and the simple count technique for staining were used for estimating the number of viable cells. The proportion of survival was utilized for estimating the ethanol tolerance, which was classified into three categories: extremely tolerant (>50 per cent survival), moderately tolerant (25–50 per cent survival), and mildly tolerant (<25 per cent survival) (Banerjee *et al.*, 2018).

Alcohol Dehydrogenase Activity (ADH) (Longhurst *et al.*, 1990)

A) Making cell extracts (with the Rude enzyme)

Before the cells broke, the 48-hour-old broth cultures of the chosen yeast isolates were collected by centrifugation at 8000 rpm for 8 minutes. The cell pellets were then twice cleaned with 10 mM potassium phosphate buffer (pH-7.5) containing 2 mM EDTA and kept at -20oC. Samples were prepared by sonicating cell preparation with glass beads (0.7 mm diameter) at 0oC for 2 min (133 V, 0.5 repeating cycles per s), after being maintained at room temperature and cleaned. Debris and intact cells were eliminated by centrifugation at 40°C for 20 minutes at 12000×g. In order to estimate protein, purified cell extracts were utilized as crude enzymes (Vuralhan et al., 2003).

B) Enzyme Assay

Crude enzyme preparations of the productive cells of yeast isolates were evaluated for ADH activity with just minimum changes. The components of the standard assay combination were crude enzyme (0.8 ml), pH 9.6 (1.5 ml), 2.0 M ethanol (0.5 mL), 0.025M NAD (1.0 ml), and 0.1M sodium pyrophosphate buffer. At room temperature (25 oC), the absorbance at 340 nm increased for three to four minutes. The initial linear portion of the curve was used to calculate absorbance (340nm/min).

The quantity of enzyme required to decrease one micromole of NAD+ per minute at 25 °C is known as an enzyme unit.

$$\text{ADH units/mg protein} = \frac{A340/\text{min}}{6.22X\,\text{mg}\,\frac{\text{protein}}{\text{mL}}\;\text{reactionmixure}}$$

Molecular characterization (Kumar *et al.*, 2018)

18S rRNA sequencing was used to determine the identity of the chosen efficient yeast isolate. A single band of high-molecular-weight DNA was visible on a 1.0% agarose gel. An 18S rRNA gene fragment was amplified using NS1 and NS4 primers. Upon agarose gel resolution, a solitary, unique 1050 bp PCR amplicon band was observed. The PCR amplicon was purified to get rid of contaminants. Using the BDT v3.1 Cycle sequencing kit and NS1 and NS4 primers, forward and reverse DNA sequencing reactions of PCR amplicons were carried out on the ABI 3730xl Genetic Analyzer. From forward and reverse sequence data, an aligner program was used to create a consensus sequence for the 18S rRNA gene.

The 'nr' database of the NCBI GeneBank database was searched using the 18S rRNA gene sequence using BLAST. The distance matrix and phylogenetic tree were produced using MEGA 10 after the first ten sequences were selected based on their largest identity score and aligned using the multiple alignment software program Clustal W (Tamura et al., 2004; Kumar et al., 2018).

III. RESULT AND DISCUSSION

Isolation and molecular characterization of *Candida tropicalis*

The mixed fruit wastes were collected from shops from different locations of Chidambaram town, Cuddalore district, Tamil Nadu. The substrate was prepared and fermented for two weeks, for the isolation of bioethanol producing yeast isolates. The isolated yeast cultures were examined through morphological and biochemical characterizing methods (Table-1). The five different yeast isolates were further subjected to utilization of different carbon sources absorption and fermentation involving lactose, glucose, galactose, and maltose and saccharose. The results revealed that all the yeast spp. tested were able to utilize and assimilate tested carbon sources except lactose.

Furthermore, 18S rRNA sequencing was used to identify Yi-2. The 18S rRNA sequence's NCBI BLAST search showed that Yi-2 and the genus Candida are closely related. A thorough phylogenetic analysis (MEGA 10) was carried out, closely related sequences were aligned, and a neighbour-joining tree was built using MEGA 10 in order to further support this discovery. The Yi-2 has proven to be a substantial sequence homolog of Candida,18S, and has formed a subclade. The rRNA sequence submission with accession number (ON258632) was received by the NCBI gene bank.

Thermal, Osmotic stress tolerance and ADH levels in particular yeast spp.

The Thermal tolerance, osmotic tolerance and ADH levels in particular chosen five yeast spp. was investigated, and the findings are shown in (Fig-3). All the five yeast isolates were shown to have high thermal tolerance The results revealed that, there was a reduction in the survival of yeast spp. as the temperature was increasing. Among the five isolates the yeast spp. *viz.*, *Candida tropicalis* Yi-2 recorded the highest population of 49 CFU/ml and also observed that two different yeast spp. could not survive under a temperature of 60ºC.

The isolate Yi-2 has the highest population survival rate (5.28 log10 CFU/ml). Among the five isolates, Yi-2 had the highest ADH activity (10.25 U mg^{-1}) while comparing with other strains. Based on the studies on their stress tolerance, the yeast isolate Yi-2 recorded maximum stress tolerance and enzyme activity, was

selected as the efficient yeast species and used for further studies.

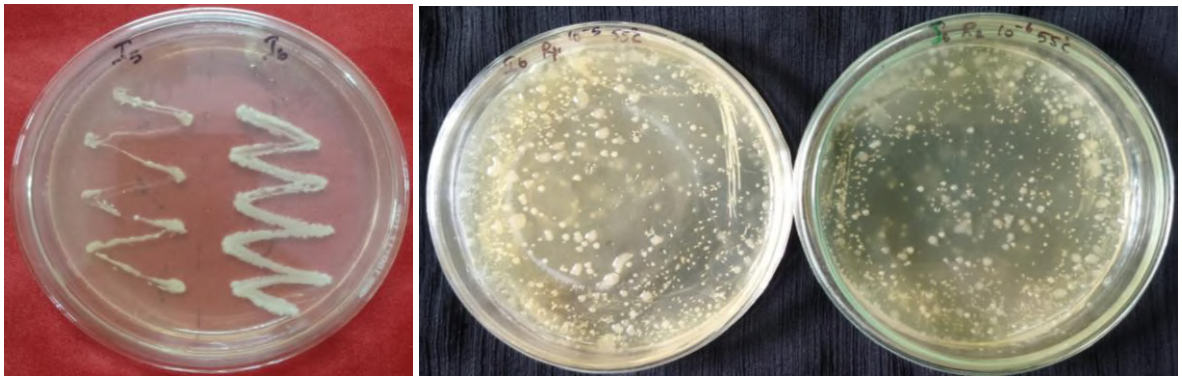
Exogenous Yeast's ability to withstand ethanol

The tolerance to exogenous ethanol of the five selected yeast spp. was examined, and the findings are shown in (Fig-3). It was discovered that all of the selected yeast isolates displayed exogenous ethanol tolerance up to 25% with variance in their efficiency. The isolate Yi-2 had the highest survival rate at all levels of ethanol concentration, namely 5%, 10%, 15%, 20% and 25%, while comparing to all other yeast isolates respectively.

Culture of *Candida tropicalis* maintained in MYA Agar medium

Efficiency tests

Ethanol tolerance test **Thermal tolerance test (after plating)**



Recovered Bioethanol from different fruit and fruit juice waste

Table 1. Morphology and Biochemical characterization of different yeast isolate

Isolate designation	Colour	Texture	Margin	Consistency	Shape	Size (µm)	Multilateral Budding	Pellicle	Ascospore Formation	Urease	Sugar fermentation	Flocculation	Pseudohypha	Tentative Identification
Yi-1	Ointment	Flush	Convex	Mucilaginous	Round to oval	4.0×8.0	+	-	+	-	+	+	+	Candida sp.,
Yi -2	Ivory/ actromic	Flush	Convex	Mucilaginous	Oval to elongate	3.25×1.6	+	-	+	-	+	-	+	Candida tropicalis
Yi -3	Ivory	Flush	Lobiform	Mucilaginous	Oval to elongate	2.74×1.28	+	-	+	-	+	-	+	Candida sp.,
Yi -4	Milky-white	Flush	Unbroken	Mucilaginous	Oval	3.77×1.53	+	-	+	-	+	+	+	Candida albicans
Yi -5	Ointment	Flush	Lobate	Mucilaginous	Round to oval	4.0×8.0	+	-	+	-	+	-	+	Candida sp.,

Table 2. Utilization of various carbon sources by different yeast Isolates

Isolate Designation	Fermentation sugar					Assimilation of sugar				
	Glucose	Galactose	Maltose	Lactose	saccharose	Glucose	Galactose	Maltose	Lactose	saccharose
Yi-1	+	+	+	-	+	+	+	+	-	+
Yi-2	+	+	+	-	+	+	+	+	-	+
Yi-3	+	+	+	-	+	+	+	+	-	+
Yi-4	+	+	+	-	+	+	+	+	-	+
Yi-5	+	+	+	-	+	+	+	+	-	+

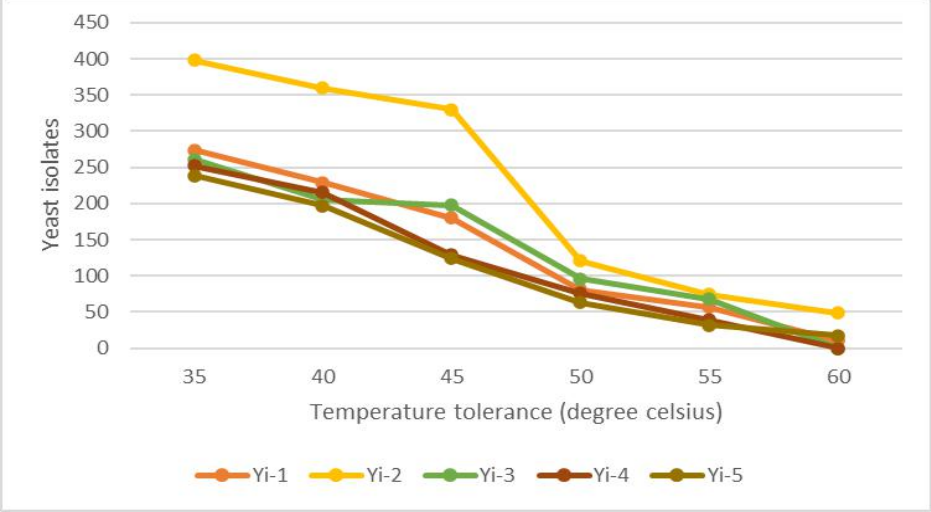


Fig.1 Temperature tolerance of selected yeast spp.

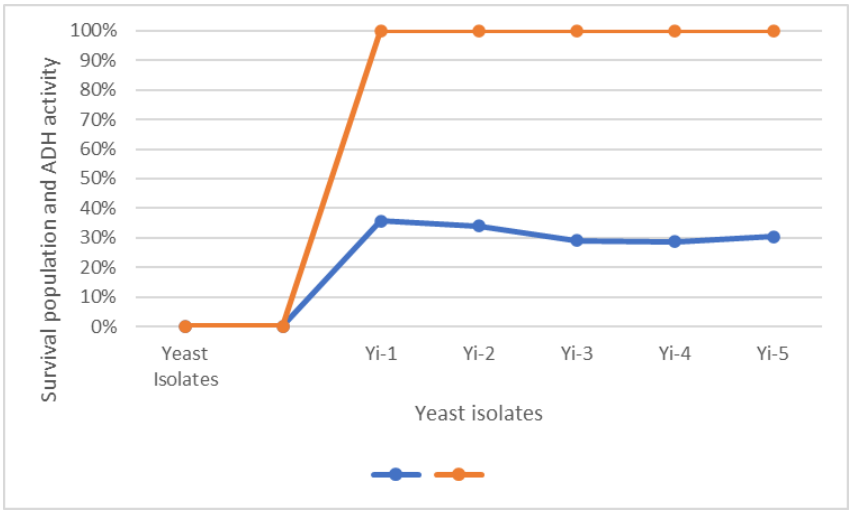


Fig.2 Osmotic stress tolerance and ADH activity of selected yeast spp.

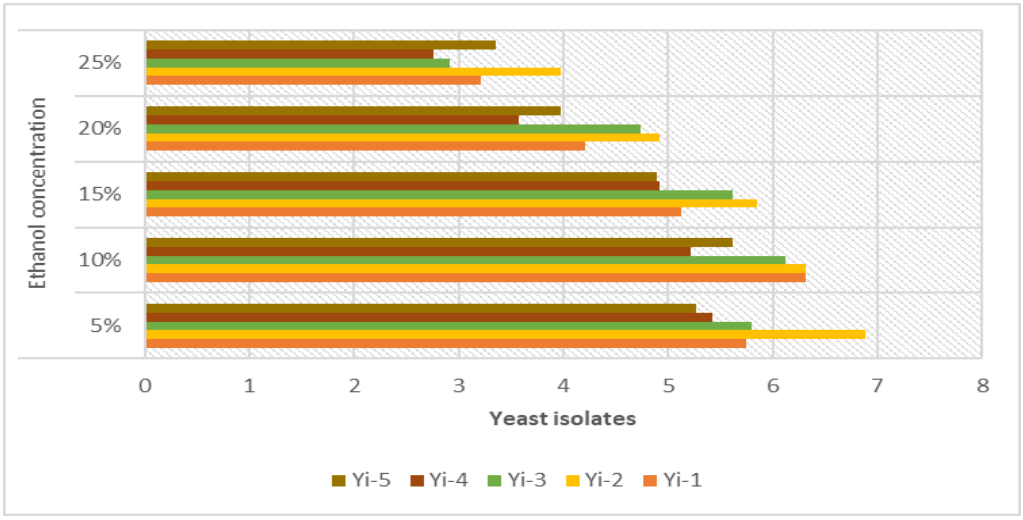
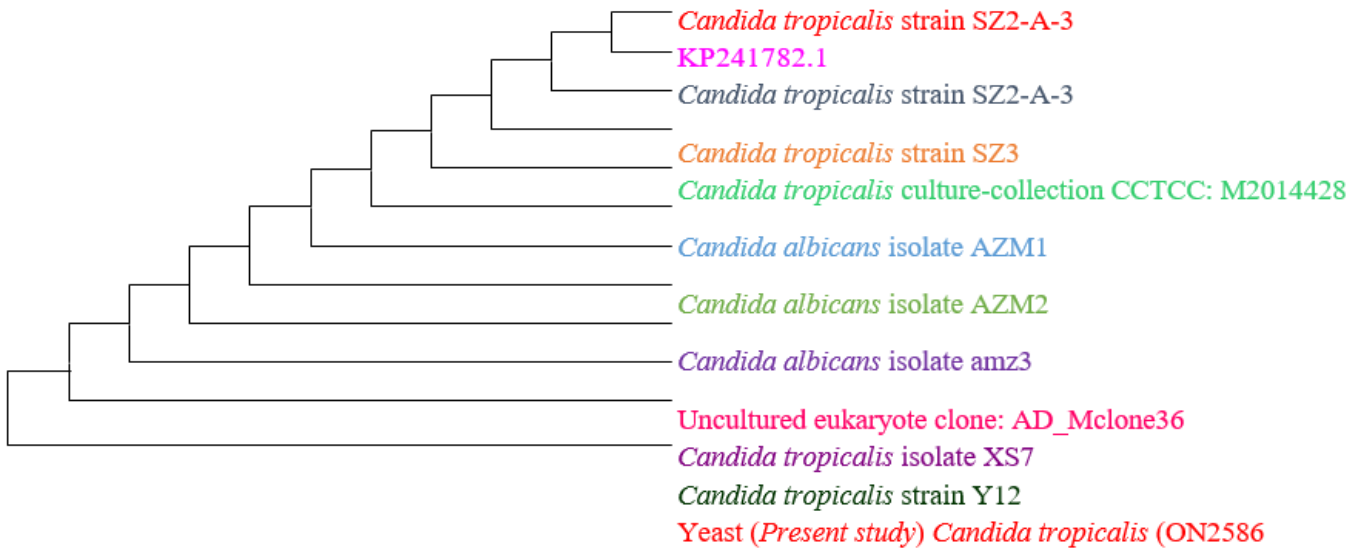


Fig.3 Exogenous ethanol tolerance of yeast spp.

Nucleotide homology and phylogenetic tree analysis



Candida tropicalis

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TAAAATCCCGACTGTTTGGAAGGGATGTATTTATT
AGATAAAAAATCAATGTCTTCGGACTCTTTGATG
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CTGGCGATGGTTCATTCAAATTTCTGCCCTATCAA
CTTTCGATGGTAGGATAGTGGCCTACCATGGTTTC
AACGGGTAACGGGGAATAAGGGTTCGATTCCGGA
GAGGGAGCCTGAGAAACGGCTACCACATCCAAG
GAAGGCAGCAGGCGCGCAAATTACCCAATCCCGA
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TCTTAACCATAAACTATGCCGACTAGGGATCGGT
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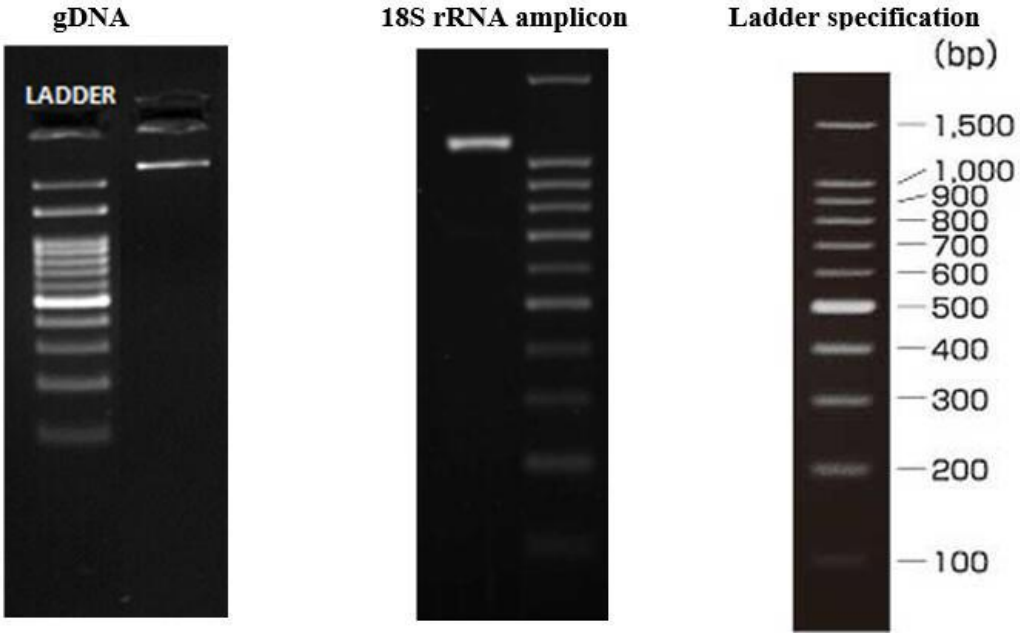
GGTCGCAAG

gDNA and 18S Amplicon QC data:



IV. CONCLUSION

To conclude the present study, the bio-ethanol production from mixed fruit waste is one of the best choices of meeting the energy requirement. Fruit waste is, better substrate for microbial fermentation because it includes starch and sugars, which can be effectively transformed into bioethanol. *Candida tropicalis* (ON258632) used in the current study was superior to other strains for fermentation of fruit wastes, since, it produced both enzymes required for hydrolysis and fermentation; it is also concluded that, by using *Candida tropicalis* Yi-2 saccharification step can be skipped in bioethanol produced from fruit wastes.

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