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Ethanol production from pulverised pawpaw peels with a consortia of microbes

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ABSTRACT

The global demand for sustainable and renewable energy sources has propelled research into bioethanol production from various biomass sources. This study investigates the production of ethanol from pawpaw (*Carica papaya*) peels using consortia of microbes. We collected and analysed soil and palm wine using standard microbiological techniques to isolate and identify microbes. Conventionally, organisms identified were *Bacillus* sp., *Aspergillus* sp. and *Saccharomyces* sp. The isolates were screened for key biochemical traits essential for ethanol production. The mould exhibited robust enzymatic activity through the production of amylase, which breaks down polysaccharide into fermentable sugars. The ethanol tolerance of the bacterium and fungi was assessed for viability and efficiency in fermentation. Pawpaw peels were collected, washed, dried and pulverised. The pulverised samples were thermally pre-treated and subjected to acid hydrolysis (10% of 0.2 M sulphuric acid) to breakdown the complex carbohydrates into fermentable sugars. The resulting hydrolysate was then subjected to fermentation with various microbial consortiums. The fermentation parameters were carefully controlled, with a 10 mL inoculum volume, pH of 5.5, temperature of 35 °C, and fermentation duration of four days. Results indicated that the highest ethanol concentration (15.2%) was achieved with the consortium of *Saccharomyces cerevisiae*, *Bacillus subtilis,* and *Aspergillus niger*, producing a significant ethanol concentration compared to the control and other microbial combinations. This study shows that the combination leveraged the synergistic effects of fungi and bacterium, enhancing the overall fermentation efficiency. These findings contribute to the growing body of knowledge on bioethanol production from agricultural waste and offer a promising pathway for sustainable energy development, thus fulfilling the Sustainable Development Goals (SDGs) seven. Integrating bioethanol production with existing agricultural practices could provide a dual benefit.

Keywords: Ethanol, pawpaw peels, microbial consortia, biofuel.

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INTRODUCTION

As the world grapples with rising energy demands and increasing environmental concerns, the search for sustainable energy sources has never been more urgent. With global population growth and industrialisation, energy consumption has soared, putting immense pressure on finite fossil fuel reserves. As of 2023, fossil fuels supply about 73-81 % of the world's primary energy demand (International Energy Agency, 2023; Resources for the Future, 2024), but due to rapid usage, their reserve will be finished in the near future. Increasing concern about climate change and the depletion of fossil fuels has spurred the search for sustainable and eco-friendly energy sources, as burning fossil fuels contributes significantly to pollution and carbon dioxide levels. Biofuels are renewable fuels that are made from biomass. Biomass are materials from living or recently living organisms (Wyman et al., 2019). Because of its advantageous effects on the environment, bioethanol is one of the most intriguing biofuels. Its impact is lessened when compared to fossil fuels because bioethanol has a smaller carbon footprint and contains oxygen (Buratti et al., 2018).

Bioethanol has attracted worldwide attention because of its potential use as an alternative automotive fuel, coupled with the fact that it is renewable. Bioethanol is a result of the fermentation of sugar-rich source employing different types of yeast and bacterial cells. Bioethanol production primarily relies on feedstocks such as sugar cane, corn and other agricultural products. However, the competition with food supply and high production costs remains significant challenges (Siddique et al., 2024). Bioethanol can be produced from lignocellulosic materials, such as fruits and vegetable waste, agro-residues, etc (Zabed et al., 2017). Fruit wastes are a rich source of natural sugars. Large amounts of fruits are consumed on a regular basis as health supplements and as foods. The easily available and cheap source for the production of bioethanol is fruit waste, which offers a sustainable way to utilise agricultural waste and reduce environmental pollution.

Paw-paw (*Carica papaya*) peels are a promising, yet underutilised, agricultural waste material rich in fermentable sugars, making them ideal for bioethanol production (Mankar *et al*., 2021). The conversion of these sugars into ethanol occurs through a biological process called fermentation, which is carried out by microorganisms. During fermentation, enzymes break down complex carbohydrates present in the pawpaw peels into simple sugars, which are subsequently metabolised into ethanol and carbon dioxide under anaerobic conditions (Siddique et al., 2024). The core bioreaction involved in bioethanol production is the conversion of glucose into ethanol and carbon dioxide via the glycolytic pathway, followed by alcoholic fermentation. Microbial fermentation is the cornerstone of bioethanol production, and the use of microbial consortium can significantly enhance the efficiency of this process.

A consortium is a group of different microorganisms that work together to perform a specific function, such as bioethanol production. In the context of bioethanol production, microbial consortia can consist of different yeasts, moulds, and bacteria, each playing a specific role in the fermentation process. In this study, a microbial consortium consisting of *Saccharomyces* cerevisiae (a yeast), *Aspergillus niger* (a mould), and *Bacillus subtilis* (a bacterium) is used to enhance the efficiency of sugar breakdown and ethanol conversion. *Saccharomyces cerevisiae* is known for its robust ethanol-producing capabilities, while *Aspergillus niger* produces enzymes such as amylases and cellulases, which break down complex polysaccharides into simple sugars. *Bacillus subtilis,* known for its tolerance to harsh conditions, can aid in reducing contamination and improving overall fermentation efficiency (Hashem et al., 2021). Studies have shown that bioethanol yields from fruit waste, including pawpaw peels, can range from 60 – 85 % of theoretical yields depending on the fermentation conditions (Zhao et al., 2020). The choice of microorganisms that is efficient and effective for bioethanol production has its own problem, as not all microorganisms that have good fermentative and hydrolytic ability are suitable for breaking down complex sugars and fermenting the simple sugar for bioethanol production. This study therefore investigated the potential of microbial consortiums in the production of bioethanol from pawpaw peels.

MATERIALS AND METHODS

Collection of samples

The local beverage (palm wine) used in the research for the isolation of *Saccharomyces cerevisiae* was obtained from Nembe Waterside (4°45′31" North latitude and 7°1′21" East longitude) in Port Harcourt, Rivers State. The soil sample used for the isolation of *Aspergillus niger* and *Bacillus subtilis* was obtained from behind the Department of Microbiology, Rivers State University (RSU). Ripened and over ripened pawpaw (*Carica papaya*) peels were collected randomly from a fruit market, Kaduna Street, D-Line, Port Harcourt, Rivers State, Nigeria.

Isolation of microorganisms

Isolation of Bacillus subtilis

One gram of the soil sample was suspended in 9 mL of sterile normal saline. A ten-fold serial dilution was carried out by transferring 1 mL from the stock into test tubes containing sterile 9 mL normal saline (Prescott et al., 2011). This was done serially to obtain 10^{-5} dilutions. Aliquots (0.1 mL) of 10^{-5} dilution were inoculated on the surface of a nutrient agar (NA) plate in duplicates and spread using a sterile bent glass rod. The inoculated plates were incubated aerobically at 37°C for 24 hours and examined for the appearance of colonies after incubation. The isolates were further subcultured on sterile nutrient agar plates and incubated at 37°C for 24 hours in order to obtain pure isolates (Bilyartinus and Siswanto, 2021). The pure isolates were maintained on nutrient agar slants and stored at 4°C for further use.

Isolation of Saccharomyces cerevisiae

Saccharomyces cerevisiae was isolated from a local fermented beverage (palm wine) using the spread plate method (Prescott et al., 2011). Aliquot (0.1mL) of 10^{-1} and 10^{-2} dilutions of the beverage was aseptically placed on the surface of a solidified Sabouraud Dextrose Agar

(SDA) plates in duplicates and evenly spread with a sterile bent glass rod. The inoculated plates were incubated in inverted position at 27°C for 48 hours. Colonies suspected to be yeast based on their colonial morphology was sub-cultured onto sterile SDA plates and incubated at 27°C for 48 hours in order to obtain pure isolates. The pure isolates were maintained on SDA slants and stored at 4°C for further use (Amadi, 2016).

Isolation of Aspergillus niger

One gram of the soil sample was transferred into a labelled test tube containing 9 mLs of sterile normal saline. The mixture was then subjected to a ten-fold serial dilution to a dilution of 10^{-3}. Aliquots (0.1mL) of 10^{-2} dilution were spread on the surface of a sterile solidified Sabouraud Dextrose Agar (SDA) plate supplemented with tetracycline in order to suppress bacterial growth and incubated at 27°C for 5 days (Prescott et al., 2011). After incubation, *Aspergillus niger* was selected on the basis of their cultural and morphological characteristics. The isolates were further subcultured to sterile SDA plates and incubated at 27°C for 5 days in order to obtain pure isolates (Amadi, 2016). The pure isolates were later maintained on SDA slants and stored at 4°C for further use.

Characterisation of microbial isolates

Identification of bacterial isolates by cultural technique

The pure bacterial isolates were exposed to biochemical assays to aid in identification, as detailed by Cheesbrough (2006). The results of the tests were entered into the search dialogue of the online bio-database software "Advanced Bacterial Identification Software (ABIS)" at https://www.tgw1916.net/bacteria_logare.html, revealing the presumed identity of all isolates.

Identification of fungal isolates by cultural technique

The fungal isolates were identified based on morphological and microscopic characteristics such as colony growth pattern, conidial morphology, and pigmentation (Douglas and Robinson, 2019). The pure isolates suspected to be *Aspergillus* sp. and *Saccharomyces* sp. were viewed with a microscope by placing a drop of lactophenol cotton blue on a clean slide. Using a sterile wire loop, a colony from the representative fungi cultures was emulsified on the lactophenol cotton blue. Then a clean cover slip was gently placed and pressed down to evenly spread the sample and eliminate air bubbles. The slide was observed under the light microscope using ×10 and x40 objective lenses (Guimaraes et al., 2006). The morphological characteristics and appearance of the fungal isolates seen were identified in accordance with the standard scheme for identification of fungi by Fisher et al. (2023).

Screening for alpha-amylase production

The fungal isolates were tested for production of amylase by starch hydrolysis in order to check if the fungal isolates have amylase production potential, which is essential for the breakdown of complex sugar contained in the pawpaw peels to be used. Modified starch agar medium consisting of soluble starch (2 g), peptone (2 g), yeast extract (1 g) and agar (2 g) was used, and the isolates were inoculated and incubated for 72 hours at 27°C. After incubation, the plates were flooded with iodine solution and observed for blue-black colour around colonies to change to brown or milky colour, which accounts for their ability to digest the starch and thus indicates the presence of alpha-amylase. Isolates with large zones of clearance were subcultured and maintained on fresh sterile Sabouraud dextrose agar (SDA) slant at 4°C for further studies (Amadi, 2016).

Ethanol tolerance test

The isolates were subjected to 10%, 15%, and 20% of ethanol by dispensing 10 mL, 15 mL, and 20 mL of ethanol into conical flasks containing 90 mL, 85 mL, and 80 mL of peptone water, respectively. The test organisms were each inoculated into this prepared broth medium and incubated at 37°C for 48 hours for the bacterial and at 27°C for 48 hours for the fungal. The ethanol tolerance test result was read using the plate count method to determine the level of growth (Breisha, 2010). The isolates that are more tolerant are further used for the fermentation. The basic step to the production of bioethanol is represented in Figure 1.

Preparation of pawpaw peel flour substrate

The collected pawpaw peels were washed with distilled water to remove any dirt and contaminants. The clean peels were then chopped into pieces. It was then sun dried for 14 days, before being ground to fine powder then packed in an airtight container, and stored until used in the fermentation process (Ajay et al., 2014).

Pre-treatment of pawpaw peel flour

Pawpaw peel powder (20 g) was soaked in 300 mL of

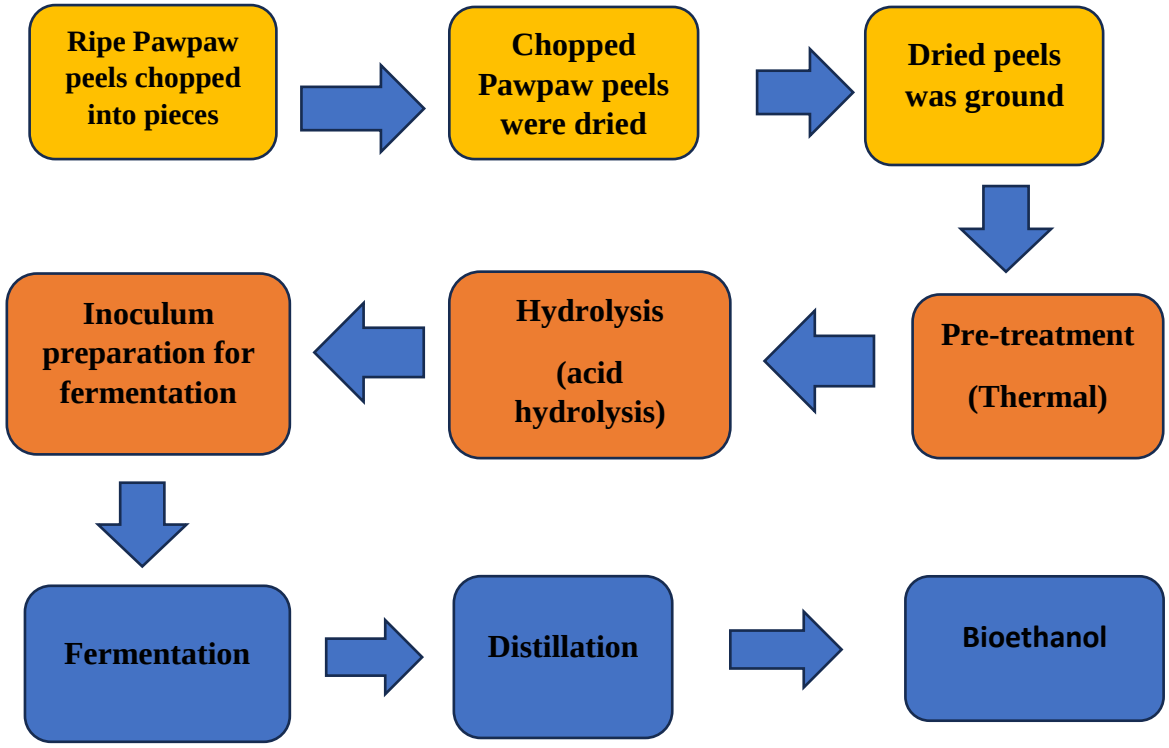

Figure 1. Flow chart of ethanol production from pawpaw peels.

distilled water for 30 minutes and then autoclaved at 121°C for 15 minutes. The thermal pre-treated samples were allowed to cool down to room temperature. After cooling, the extract was filtered using a muslin cloth to get a pure solution.

Hydrolysis of pre-treated pawpaw peel flour

Ten percent (10%) of 0.2 M of sulphuric acid was prepared and mixed with the pretreated solution. Sulphuric acid was used with the filtrate in the ratio of 6:1, then the solution was heated to 121°C for about 6 hours and then allowed to cool. The pellets were discarded and the pH of the hydrolysate was adjusted. The sugar content of the hydrolysate was analysed using Fehling's method.

Test for reducing sugar

The presence of reducing sugars was assayed using Fehling's qualitative method. Fehling's solution was prepared by mixing equal volumes (10 mL each) of Fehling's solution A and B in a beaker. Five millilitres of the solution were added into a test tube. Little quantities of the hydrolysate were added into the test tube, after which the mixture was kept in a boiling water bath for about 15 minutes. The change in colour from blue to green indicated the presence of reducing sugar (Vinotha et al., 2023).

Inoculum preparation

The inoculum of *Aspergillus niger*, *Bacillus subtilis* and *S. cerevisiae* was prepared from their slant cultures. *A. niger* and *S. cerevisiae* were inoculated on freshly prepared Saboraud Dextrose Agar (SDA) and incubated at 27°C for 48 hours (*S. cerevisiae*) and 72 hours (*A. niger*). *Bacillus subtilis* was cultured on nutrient agar and incubated at 37°C for 24 hours. After incubation, the isolates were inoculated into 200 mL of sterile peptone water and incubated for 48 hours for *Bacillus subtilis* and *Saccharomyces cerevisiae*, while *A. niger* was incubated for 72 hours (Ado et al., 2009).

Experimental design

The hydrolysate was used in preparing the experimental setup. The experimental set-up consisted of eight treatments, including the control, in an airtight container with an outlet (for the release of CO_2). Fermentation was carried out at pH 5.5 for 4 days under static conditions at

35°C. The experimental setup is presented in Table 1.

Determination of ethanol content

Ethanol concentration was determined by measuring its specific gravity after distillation; the specific gravity values obtained were used to determine ethanol concentration from an ethanol standard specific gravity curve prepared using known concentrations of pure ethanol as adopted by Charanchi et al. (2018).

Distillation

The fermented liquid was transferred into a round bottom distillation flask and placed on a heating mantle attached to a distillation column with running tap water. Another flask was fixed to the other end of the distillation column to collect the distillate at 78°C (Oyeleke et al., 2012).

Table 1. Experimental setup.

Cultured isolates	Initial volume (mL)	Culture type	Volume of inoculum (mL)	Final volume (mL)
None	300	Control	0	300.0
Bac	300	Monoculture	10	310.0
Sac	300	Monoculture	10	310.0
Asp	300	Monoculture	10	310.0
Sac + Bac	300	Consortium	5 + 5	310.0
Sac + Asp	300	Consortium	5 + 5	310.0
Bac + Asp	300	Consortium	5 + 5	310.0
Sac + Bac + Asp	300	Consortium	3.3 + 3.3 + 3.3	309.9

Keys: Bac - *Bacillus subtilis,* Asp – *Aspergillus niger,* Sac – *Saccharomyces cerevisiae.*

Specific gravity

A clean and dry specific gravity bottle was weighed with its stopper. It was filled with distillate and weighed, and the specific gravity was calculated as:

$$\text{Specific Gravity (g/cm}^3\text{)} = \frac{W2 - W1}{\text{Volume of distillate}}$$

Where
W1 = empty weight of specific gravity bottle
W2 = Weight of sample + specific gravity bottle.

Confirmatory test for ethanol produced using pawpaw peels

The method adopted by Jimoh et al. (2009) was adopted in the confirmation of ethanol produced using pawpaw peel. In this method, 1 mL of ethanoic acid was added to 1 mL of the distillate and then heated in the presence of concentrated sulphuric acid. The production of a sweet ester smell indicated the presence of ethanol.

Statistical analysis of data

All data in this study were expressed as mean ± standard deviation. Statistical analysis was carried out by two-way analysis of variance (ANOVA) to compute statistically significant differences at $p < 0.05$. Turkey's pairwise comparison was used to separate the means. All graphics were constructed using Microsoft Excel V22, while the statistical tool used was SPSS (v27).

RESULTS

The result showing the ethanol tolerance of bacterial and fungal isolates is presented in Figures 2 to 4. All the bacterial isolates were tolerant (able to grow) at 10%, 15%, and 20% ethanol concentrations but had an optimum tolerant range at 20% ethanol concentration (Figure 2). The moulds were all tolerant to ethanol at all concentrations but highly tolerant at the 10% concentration (Figure 3). The yeast isolates were tolerant to ethanol at concentrations of 10% and 15%, but the best tolerance was at 10% concentration, while the least was at 20% concentration (Figure 4).

Figure 3 is the ethanol standard specific gravity curve that was prepared by plotting the specific gravities of different known concentrations of ethanol against their concentrations. The density of the fermentation liquid in the different setups is presented in Table 2. The specific gravity of the ethanol yield ranged from 0.9908 to 0.9644 g/ml. The highest bioethanol yield from pawpaw peel waste was achieved with the inoculation of a consortium of *S. cerevisiae, Aspergillus niger,* and *Baccilus subtilis* with a specific gravity of 0.9644 g/ml equivalent to an ethanol concentration of 15.2% (Figure 5). The sweet ester smell perceived indicated the presence of ethanol after heating 1 ml of the distillate with 1 ml of ethanoic

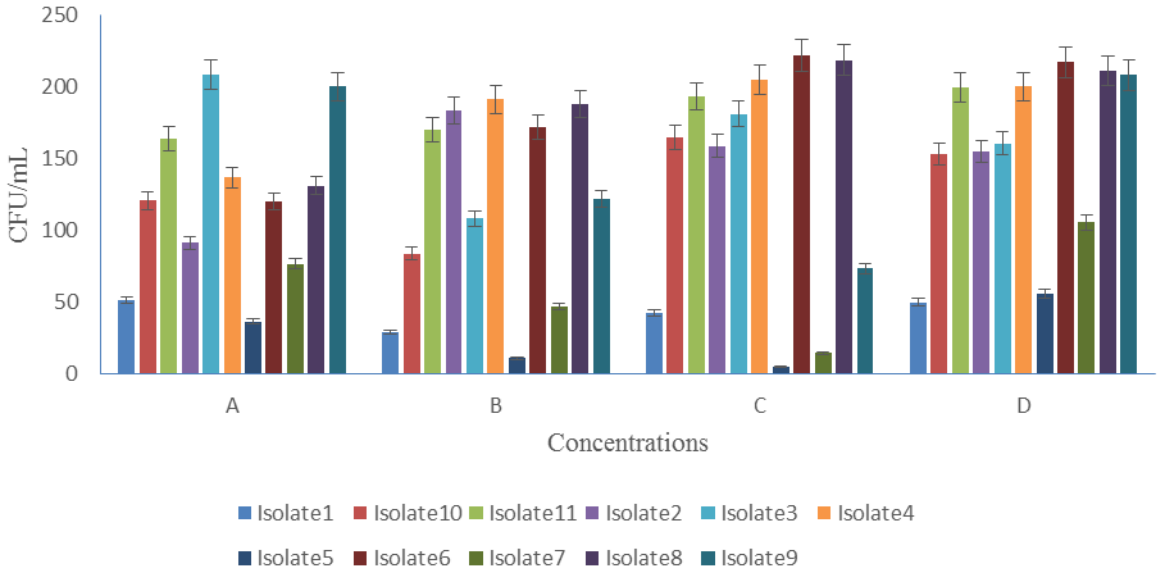

Figure 2. Ethanol tolerance test of *Bacillus* sp (isolate 6).
Keys: A = 10%; B = 15%; C = 20%; D = 0%.

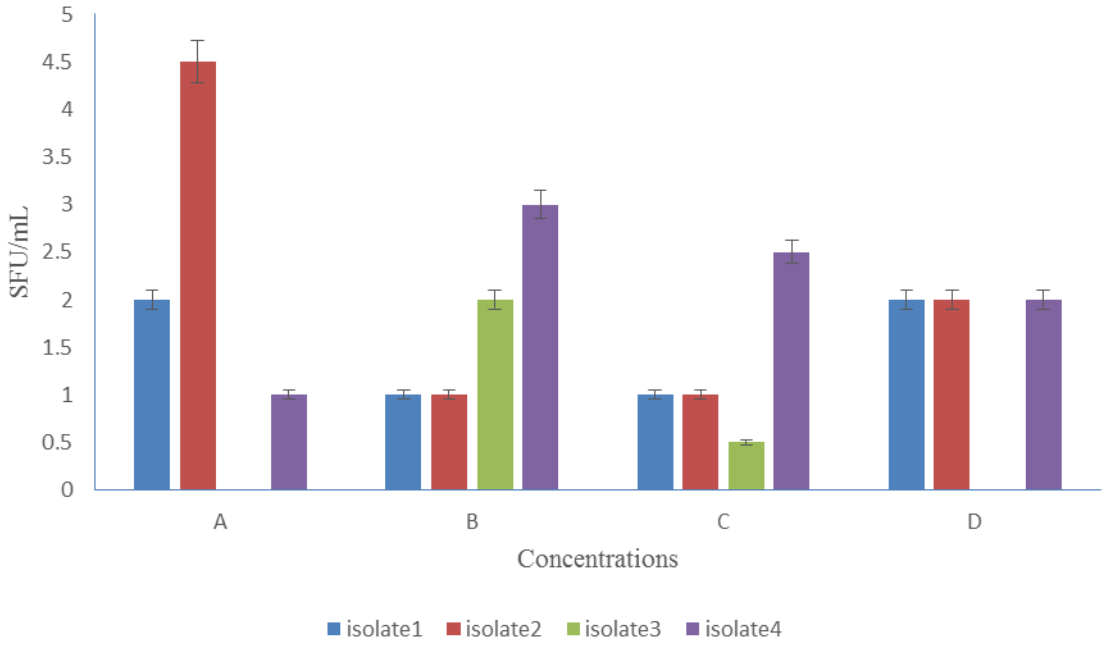

Figure 3. Ethanol tolerance test of *Aspergillus niger*.
Keys: A = 10%; B = 15%; C = 20%; D = 0%.

acid in the presence of concentrated sulphuric acid.

DISCUSSION

The fungal isolates screened for amylase production were found to have good amylase production potential, which is important in the hydrolysis of starch. This is in agreement with the work of Omemu et al. (2005), who reported that *A. niger* can be used for industrial production of ethanol, gluconic acid, and citric acid because of its hydrolytic capacities in amylase

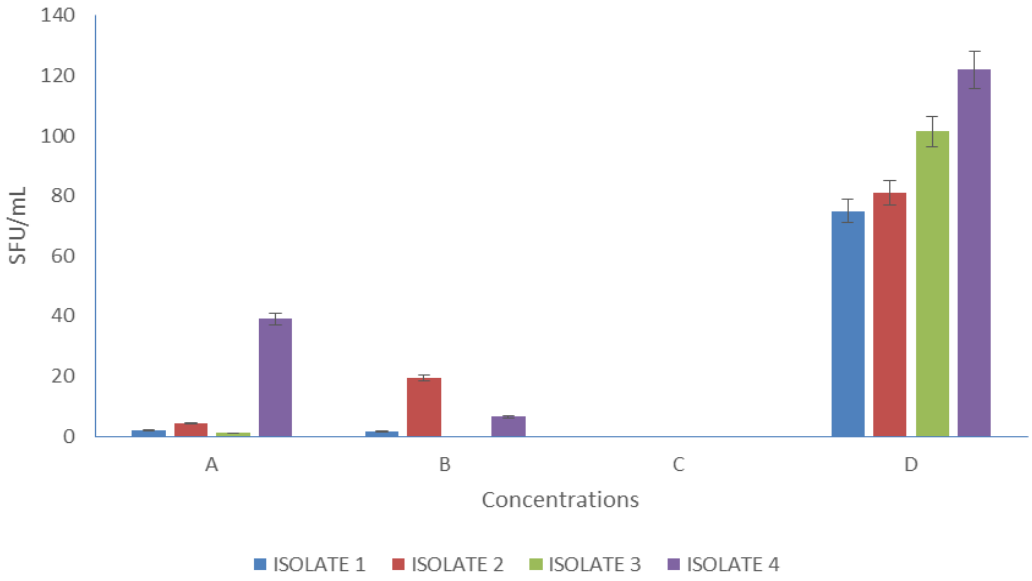

Figure 4. Ethanol tolerance test of *Saccharomyces cerevisiae.*
Keys: A = 10%; B = 15%; C = 20%; D = 0%.

Table 2. Density of the fermentation liquid from the setups.

Setup	Density (g/mL)	Ethanol concentration (%)
Pp	0.9908	3.6
Pp + Bac	0.9852	6.1
Pp + Asp	0.9808	8.2
Pp + Sac	0.9779	10.4
Pp + Sac + Bac	0.9761	11.1
Pp + Bac + Asp	0.9741	12.8
Pp + Sac + Asp	0.9722	13.7
Pp + Sac + Bac + Asp	0.9644	15.2

Keys: Pp – Pawpaw peels; Bac - *Bacillus subtilis;* Asp – *Aspergillus niger;* Sac – *Saccharomyces cerevisiae.*

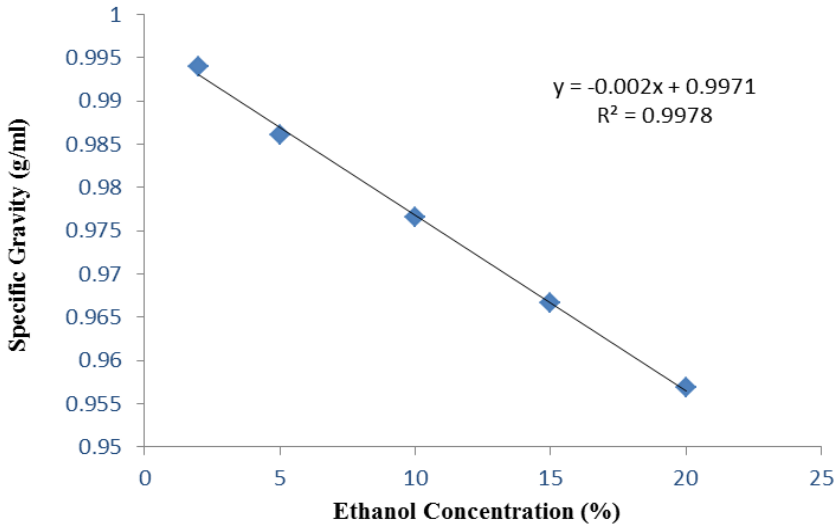

Figure 5. Ethanol standard specific gravity curve.

production. *A. niger* demonstrated significant amylase activity, as evidenced by large clear zones around its colonies. The mould isolates showed moderate ethanol tolerance. This level of tolerance is adequate for its role in hydrolysing complex carbohydrates early in the fermentation process before ethanol concentrations become inhibitory (Singh et al., 2009). The yeast isolates tolerated ethanol concentrations of 10% and 15% but had the best tolerance at 10% concentration. Growth of yeast isolates in 8% ethanol concentrations has been reported by Aminu et al. (2018). Also, previous studies have reported the growth of the yeast *Saccharomyces cerevisiae* in a medium supplemented with 20% v/v ethanol (Kumar et al., 2012; Ukponobong et al., 2018). The *Bacillus* isolates had a tolerance range of 10-20 % to ethanol.

Saccharomyces cerevisiae yielded higher ethanol concentrations than *A. niger* and *B. subtilis.* Similar findings have been reported by Balat (2011) and Al-Shorgani et al. (2011), who documented the effectiveness of *S. cerevisiae* in various fermentation processes. The control setup, consisting of pawpaw peels alone, yielded negligible ethanol, underscoring the necessity of microbial intervention for effective bioethanol production. The fermentation using *A. niger* alone yielded lower ethanol concentrations compared to *S. cerevisiae*. *A. niger* is primarily known for its enzymatic capabilities in breaking down complex carbohydrates into simpler sugars rather than directly producing ethanol. The role of *A. niger* in saccharification is well documented, as noted by Agu and Aniche (2013), who highlighted its importance in preparing substrates for fermentation by other microorganisms. Thus, while *A. niger* contributed to the initial breakdown of the pawpaw peel substrate, its role in ethanol production was limited. *Bacillus subtilis*, known for its amylase production, also yielded lower ethanol concentrations when used alone. This bacterium is effective in hydrolysing starches into fermentable sugars but is not as efficient in fermenting these sugars into ethanol compared to yeast like *S. cerevisiae*. The study by Hassan et al. (2015) supports the use of *B. subtilis* for substrate preparation rather than as a primary ethanol producer.

Combination of *S. cerevisiae* and *B. subtilis*, the ethanol concentrations improved compared to the individual organisms. This synergy can be attributed to *B. subtilis* enhancing substrate availability through hydrolysis, which *S. cerevisiae* then efficiently ferments into ethanol. Hashem et al. (2021) demonstrated the benefits of such mixed cultures in optimising ethanol production, strengthening the validity of this study. The consortium of *S. cerevisiae* and *A. niger* also showed high ethanol concentrations, benefiting from the saccharification abilities of *A. niger* and the fermentation efficiency of *S. cerevisiae*. This combination was effective in maximising ethanol production, as noted by Saha et al. (2013) and corroborated by Agu and Aniche (2013), who found similar benefits in using such microbial partnerships. The combination of *B. subtilis* and *A. niger* focused on maximising the hydrolysis of the substrate. While this combination improved the availability of fermentable sugars, the overall ethanol concentration was lower than those involving *S. cerevisiae* and *Aspergillus niger*. This result underscores the importance of having a potent fermenter in the consortium, a conclusion supported by the findings of Balat (2011).

In this study, the highest ethanol concentration was achieved with the consortium of *S. cerevisiae*, *Bacillus subtilis* and *A. niger*. This combination leveraged on the comprehensive breakdown of complex carbohydrates by *A. niger* and *B. subtilis* followed by efficient fermentation by *S. cerevisiae*. Research by Zhao et al. (2020) supports the use of multi-microbial consortia for optimising bioethanol production, demonstrating enhanced ethanol efficiency and yield. Recent studies have also demonstrated that the synergistic use of hydrolytic microbes alongside ethanol-producing yeasts can significantly improve bioethanol yield by optimising the availability of sugars for fermentation (Persson et al., 2020; Zhu et al., 2023). *Saccharomyces cerevisiae* has long been recognized for its ability to ferment hexose sugars like glucose through glycolysis. Whereas *Aspergillus niger* and *Bacillus subtilis* improve the availability of fermentable sugars, thus enhancing the overall efficiency of the process. From the research, the density value obtained is still far from the bioethanol density standard due to the distillation process. The study still uses simple distillation and a manual temperature indicator, so the possibility of the temperature exceeding the boiling point of the ethanol increases and makes water enter the product.

CONCLUSION

The production of bioethanol from pawpaw peels with consortia of microorganisms was investigated. The consortia of *Saccharomyces cerevisiae*, *Bacillus subtilis* and *Aspergillus niger* showed superior performance in ethanol production compared to individual microorganisms or other consortia. The study also revealed that co-metabolism and complementary activities of microbes resulted in higher ethanol production. The consortium of *A. niger, S. cerevisiae* and *B. subtilis* is preferrable since it had a better yield of ethanol than using them singly. This implied that these isolates displayed synergism in producing a higher ethanol yield. Thus, *S. cerevisiae* is known for its sugar fermentative ability; *B. subtilis* and *A. niger* are known for the breakdown of starch into usable sugars. The findings of this study support the potential of microbial consortia in enhancing bioethanol production from agricultural

residues, offering a sustainable and economically viable solution to energy needs and waste management challenges. Further research should investigate the optimal ratios of the different microbial strains used in consortia to maximise ethanol yields. Fine-tuning these ratios could enhance the efficiency of the bioethanol production process.

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