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Technological Characterization and Identification of Potential Yeast Starters from *Diospyros mespiliformis* Fruit Pulp

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Abstract The aim of this study was to add value to *Diospyros Mespiliformis* fruits from northern Côte d'Ivoire. Yeasts were isolated from *Diospyros Mespiliformis* pulp and screened to select potential starters. Following screening, the best strains were identified using the Maldi Tof technique to determine the species involved. The results enabled us to select three (3) strains with the best growth rates compared with the other strains tested. These strains belong to the species *Candida krusei*, *Candida guillermondii* and *Candida fermentati*. These strains could thus be used as potential starters in biotechnological applications. *Diospyros Mespiliformis* would be of interest in the development of yeast culture collections useful for controlled fermentations.

Keywords: *Diospyros Mespiliformis, isolation, screening, starters, yeasts*

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1. Introduction

Fruit plays an important role in the daily lives and well-being of many people. In many countries, people prefer cultivated fruit to wild fruit [1]. Wild fruit trees are defined as all woody fruit species growing in the wild [2]. These species play a vital role in the daily lives of rural populations. This is reflected in their use in the food and pharmaceutical industries worldwide [3]. Most of these wild fruits are eaten raw where they are harvested, while a small proportion of these fruit species are spared during land clearance and marketed for their economic profitability [4]. Ivorian soil is home to a large number of wild fruit trees, including *Diospyros mespiliformis*. This wild fruit belongs to the *Ebenaceae* family and is also known as the African ebony tree [3]. *Diospyros mespiliformis* is a multipurpose woody plant [5]. Indeed, all its parts (roots, bark, fruit, leaves) are used for various purposes [3]. Unfortunately, although this fruit has economic potential, it is poorly exploited and little known from a nutritional and industrial point of view by the Ivorian population. It is a species that produces fruit whose pulp is very sweet and much appreciated not only by the animals that ensure its dispersal in the wild, but also by rural populations [3]. Due to the carbohydrate content of its pulp, which gives it a sweet taste, *Diospyros mespiliformis* could be a suitable substrate for research into fermentative flora of interest to the food industry. Indeed, the microflora of fruit and vegetables is dominated by bacteria, yeasts and molds [6]. Processing by fermentation has been proposed and applied in some countries as an alternative for most wild fruits. Most often, the resulting product is a fruit wine, which is often distilled and has a variable alcohol concentration, or fruit vinegar [7]. During these alcohol fermentation processes, several yeasts are involving whose identity and functional properties are unknowns. Knowledge of the functional properties of these yeasts will make it possible to select starter culture who will be able to optimize the alcoholic production of the *Diospyros mespiliformis* fruit and other fruit notably cocoa fermentation [8]. The aim of this study is to contribute to the valorization of *Diospyros mespiliformis* fruits by identifying and screening yeast strains involved in the alcoholic fermentation of these fruits, in order to use them as starters for the improvement and optimization of alcoholic fermentation.

2. Materials and Methods

2.1. Material

The biological material used in this study is the fruit (pulp) of *Cola cordifolia* (Figure 1) from trees at the PELEFERO GON COULIBALY University in Korhogo (Côte d'Ivoire). The fruits have been harvested and sent to the University's laboratory for microbiological analysis.

A

B

Figure 1. Ripe (A) and unripe (B) fruits of *Diospyros mespiliformis*

2.2. Methods

2.1. Isolation and Identification of Yeasts

Diospyros mespiliformis beans (25 g) are diluted in 225 ml of peptone salt solution (0.1% (w/v) bactopeptone and 0.85% (w/v) NaCl). The solution thus prepared constitutes the stock solution, which undergoes successive decimal dilutions (10^{-1} to 10^{-4}) with tryptone salt solution. A volume of 100 µL of each dilution has been streaked onto MYGP agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L bactopeptone and 10 g/L glucose) containing 100 mg/L chloramphenicol (Sigma). After inoculation, Petri dishes have incubated and yeast strains have morphologically been identified after 3 days of incubation at 30°C, then yeast cells have been observed fresh under a precision optical microscope (Zeiss Microlmaging GmbH 37081, Germany) at objective ˣ100. Presumptive yeast isolates were stored in cryotubes containing MYGP broth supplemented with 20% glycerol at -20°C for subsequent testing [9].

2.2. Yeast Screening

Depending on the test to perform, strains are grown overnight at 25 ◦C either on MYGP agar or in MYGP broth and then the cultures are used to inoculate either MYGP broth or specific media. For the latter, each strain is harvested by centrifugation (5000 rpm for 10 min), washed once in NaCl 0.9% (w/v) solution, re-suspended to Optical Density (OD) 600 of 1.0 in the same solution [10]. Subsequently, each strain has been spotted (5 µL) in duplicate onto specific media.

2.2.1. Screening of High Fermentative-capacity Yeasts

The fermentative capacity of yeast strains isolated from *Diospyros mespiliformis* pulp has been studied according to the method of [11] with a slight modification. From pre-culture of 24 hours, pure yeasts culture has been suspended in saline tryptone to get an optical density of 0.7 at 600 nm and 100 µL of this suspension have been used to inoculate 10 mL of YPG medium containing a Durham tube into essay tube. Then, the culture has been incubated at 30°C for 6 days, without agitation. Fermentative capacity has also been determined by measuring the gas production in Durham test. Under anaerobic conditions, yeast oxidise sugars to ethanol, producing CO2. This volume of gas and ethanol produced is related to the fermentative strength of strain [12].

2.2.2. Catalase Activity

The catalase activity of yeasts was evaluated according to the method described by [8] by directly adding 3% (v/v) hydrogen peroxide onto 48 h-old yeast colonies. Catalase activity was evidenced by the formation of bubbles. In this case, results were expressed in terms of absence (−) or presence (+) of this activity.

2.2.3. Acetic Acid Production

A loopful (1 µL) of biomass of each strain has been streaked onto Chalk agar (yeast extract 3 g/L, glucose 10 g/L, calcium carbonate 3 g/L, agar 15 g/L) plates and has incubated for 7 d at 25 °C [13]. The presence and extent of a clear halo around the yeast biomass has indicated the rate of acetic acid production.

2.2.4. H_2S Production

A loopful (1 µL) of biomass of each strain was streaked onto BiGGY agar plates and incubated for 48 h at 25 ◦C [14]. The color intensitys of the biomass indicated the rate of H_2S production [15].

2.2.5. Protease Activity

Each strain culture has been spotted onto Petri plates with a medium prepared by mixing the two following

solutions: malt extract 3 g/L, yeast extract 3 g/L, peptone 5 g/L, glucose 10 g/L, NaCl 5 g/L, agar 20 g/L (separately sterilized), adjusted to pH 3.5 with 0.1 M HCl; and a skim milk solution (10% w/v) prepared and treated at 100 ◦C for 10 min. After incubation for 3 days at 25 ◦C, the presence of a clear halo around the yeast spot indicated protease activity [16].

2.2.6. Growth at Various Temperatures and pH

Yeast strains have been grown in standard liquid medium containing 0.05% yeast extract; 0.3% casein peptone; 1% glucose at pH 5.6. To assess the influence of temperature on the growth of yeast isolates, 10 ml of standard liquid medium contained in a test tube has been inoculated with 100 µL of yeast pre-culture, OD600 = 0.7. The cultures have then been incubated for 72 h at temperatures ranging from 30 to 50°C. The influence of pH variations on the growth of yeast isolates has been analyzed in the same medium at different pH values (2.5; 4; 5 and 7) and has been incubated at 30°C. Yeast isolate growth has been determined by measuring the turbidity of the culture medium at 600 nm using a spectrophotometer [8].

2.2.7. Determination of Ethanol by Distillation

An Ethanol assay has been carried out at the end of fermentation. Fermented molasse has been distilled to ethanol, using the powerful Quickfit/FC3/13 column distiller, 85 cm long and 4.45 cm in diameter (Fischer Scientific, Sweden) A temperature of 79°C has been maintained at the top of the column until all the alcohol in the fermented juice evaporated and condensed. The use of an alcoholmeter (Biobase, china) has enabled to determine Ethanol content. Three independent experiments have been performed.

2.2.8. Identification by MALDI-TOF of Potential Starters

Bacterial isolates underwent MALDI-TOF MS whole-cell analysis. This method discriminates bacteria based on screening of observed peaks as protein biomarkers for bacterial identification [17]**.** This strategy is enhanced by utilizing one or more reference strains for each species to be included in the database [18,19].

2.2.9. Statistical Analysis

All measurements have been performed in triplicate. Statistical analyses of the data have been performed through Statistica version 7.1 software. Means have been compared through Tuckey's HSD test with a significance level of only 5% ($p < 0,050$).

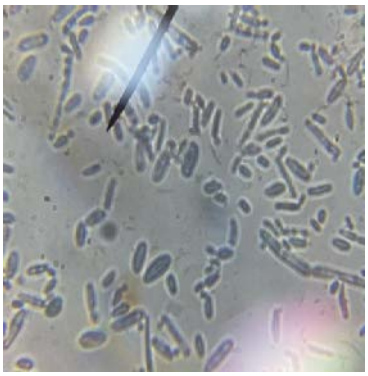
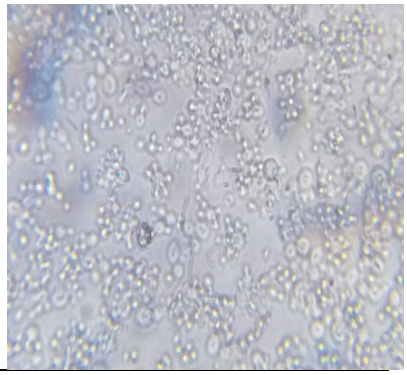
3. Results and Discussion

3.1. Results

3.1.1. Morphology of Yeast Involving in Fermentation of *Diospyros mespiliformis* Pulp

forty-six (46) yeast isolates have been isolated from *Cola cordifolia* pulp. Table 1 shows the morphology, color, and texture of the colonies of the different strains grown on MYGP agar.

Table 1. Colony characteristics of yeast strains grown on MYGP agar

Strains	Colony Morphology, Color, and Texture	Optical microscopy of representative strains
YD 1, YD 3-YD 36	Convex, Cream-colored, Smooth/Glossy	
YD 2, YD 37-YD 46	Convex, White, Matt/opaque	

3.1.2. Fermentative Capacity of Yeast Strains

Among the 46 analyzed yeast strains for the fermentative capacity, only Sixteen (9) have shown a high fermentative capacity with gas amount above to 4 cm^3. While five (6) isolates have a gas production between 1 and 4 cm3 and Thirty-Five (31) have gas production between 0 and 1 cm3 (Table 2).

Table 2. CO2 production of yeast isolate from *Diospyros mespiliformis* pulp

Fermentative capacity	Volume of CO2 (cm3)	Number of isolates
High level	[4 – 6 cm^3]	09
Middle level	[1 – 4 cm^3[	06
Low level	[0 – 1 cm^3[	31

3.1.3. Biochemical Properties of High Fermentative Capacity Yeasts Involving of *Diospyros mespiliformis* Pulp Fermentation

All the yeasts isolates involving of *Diospyros mespiliformis* pulp fermentation tested have the catalase and H_2S production capacity. But the level of production is different from one yeast isolate to another. About protease activity, six (6) have produced with high activity for three (3) isolates (YD 33, YC 37, YD 42). While only two (2) yeasts isolates (YD 19, YD 25) are able to produce acetic acid (Table 3).

Table 3. Biochemical properties of yeasts isolates involving of *Diospyros mespiliformis* pulp fermentation

strains	Acetic Acid Production[a]	Catalase activity[b]	Protease Activity[c]	H_2S Production[d]
YD 7	-	+	+	+
YD 16	-	+	-	++
YD 19	+	+	-	++
YD 21	-	++	-	+
YD 25	+	+	+	++
YD 29	-	++	+	++
YD 33	-	+++	+++	+
YD 37	-	+++	+++	+
YD 42	-	+++	+++	+

[a]Halo: -, none; +, low. [b] Development of bubbles: +, low; ++, medium, +++, high. [c]Activity: −, no halo; +, small diameter; ++, medium diameter; +++, large diameter. [d] Biomass color: ++, black brown; +, brown.

3.1.4. Growth Capacity of the High Fermentative Yeasts Isolate Involving Of *Diospyros mespiliformis* Pulp Fermentation At Different Temperature

The growth curves of the 9 yeasts strains subjected to various incubation temperature are presented in Figure 2. The selected yeast strains had their optimum growth at 35 °C unlike the Yeasts strain YD 7 and YD 21, which had their optimum growth at 35°C. The stabilization of the growth curve was obseved at 55 °C. Concerning yeast strains YD 7 and YD 21, a decrease in growth rate was observed from 30 to 45°C.

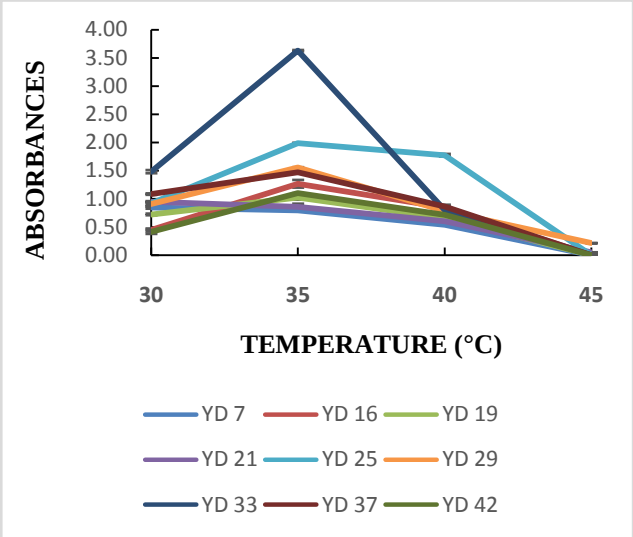


Figure 2. Effet of temperature on yeast strains growth. The observation is mean of 3 replicate experiments (n=3)

3.1.5. Impact of pH on the Growth of Selected Yeast Isolates

For pH values between 4 and 5, an increase in microbial growth was observed. The selected yeast strains had their optimum growth at pH5 (YD 37, YD 33, YD 7, YD 21, YD 29, YD 19 and YD 7) unlike strain YD7, which had its optimum growth at pH7. Beyond pH5, a slowdown in growth was observed up to pH7. Finally, it should be noted that strain YD 42 showed stable growth during pH variation (pH4 to pH7).

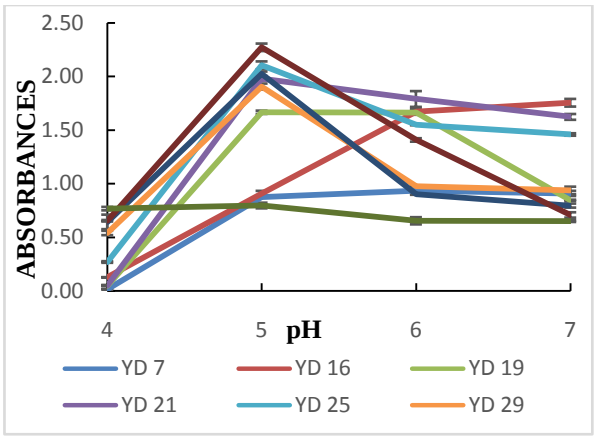
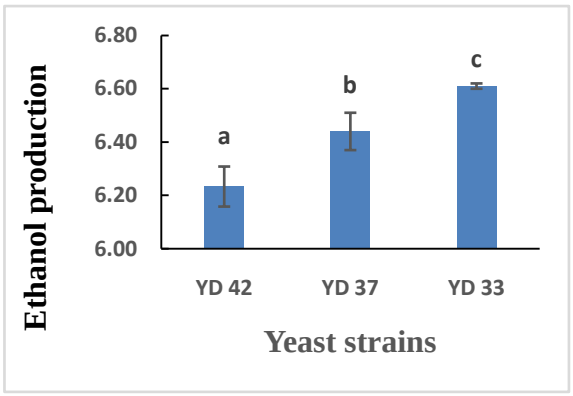


Figure 3. Effet of pH on yeast strains growth. The observation is mean of 3 replicate experiments (n=3)

3.1.6. Ethanol Production

Isolates YD 42, YD 37, and YD 33 showed the best characteristics (high CO2 production, no acetic acid production, high catalytic activity, high protease activity, low H2S production, and good resistance to temperature and pH variation) for fermentation. These isolates have been selected for ethanol production and identified. The results of the statistical analysis showed a significant difference in ethanol production. Indeed, strain YD 33 had the highest ethanol production, while strain YD 42 had the lowest.



Nb: Histograms assigned to different letters have statistically different values (Tuckey's HSD test, $p < 0.050$).

Figure 4. Ethanol production by selected yeast strains

3.1.7. Identification by MALDI-TOF of the Three Best Yeast Strains

The results of identifying the three selected strains using MALDI-TOF showed the presence of a single genus and three species: *Candida krusei* (YD 33), *Candida guilliermondi* (YD 37) and *Candida fermenti* (YD 42).

4. Discussion

The first part of this study consisted in isolating yeasts from *Diospyros mespiliformis* pulp. Thus, isolation on MYGP medium yielded 46 isolates: YD1 to YD46. The

study of phenotypic characteristics has enabled us to distinguish two colonies that have the same criteria: small, Convex, Cream-colored, Smooth/Glossy colonies with regular outlines and large Convex, White, Matt/opaque colonies with irregular outlines. These results are identical to those of [20] who states that the characteristics of pure yeast strains lie in size, color, surface appearance, contour appearance, relief, consistency and transparency. In addition, [8] isolated from *Cola cordifolia* pulp yeast strains with the same characteristics as those studied here. After purification, a study of the microscopic characteristics of the pure isolates showed that the cells of the different isolates are: elongated and oval in shape; large and small in size. These observations testify the definition proposed by [21] who distinguishes yeasts, which belong to the fungi group, by their unicellular character.

The second part of the study consisted in screening yeast isolates to select the best ones for use as starters for ethanol production. In fact, a strain's ability to offer improved technological and functional performance is a key property in its selection as a potential starter [22]. Thus, among the 46 yeasts analyzed for their fermentative capacity, nine (9) showed a high fermentative capacity with a CO_2 volume greater than 4 cm^3. These results are similar to those of [8] who showed that among 56 strains, 16 yeast strains were selected for their high fermentative capacity with a CO2 volume greater than 4 cm^3. These yeast strains are likely to produce high quantities of ethanol, since during alcoholic fermentation, the quantity of carbon dioxide (CO_2) would correspond to the quantity of ethanol produced [12].

Concerning acetic acid production, very few strains have been able to produce it. Very few strains were able to produce acetic acid. This seems very interesting, given that acetic acid is one of the compounds that have an impact on the sensory profile of wine, determining its quality. According to several authors, an acetic acid concentration of 0.7–1.1 g/L is not considered pleasant, tasteful; the maximum acceptable limit for volatile acidity in most wines is 1.2 g/L of acetic acid [23,24]. Yeast strains that produce little or no acetic acid are the most useful during alcoholic fermentation. Based on this principle, the nine strains selected in this study are interesting.

Results from the catalase test gave information about the ability of strains to cope with oxidative stress and to perform better during fermentation [25]. All of the strains tested in this study were catalase positive to various extents and in agreement with other authors [26,27]. However, the strains YD33, YC37 and YD 42 with the highest catalytic activity are the most interesting for alcoholic fermentation.

With regard to H_2S production, all the strains studied produced little or moderate amounts of hydrogen sulfide. The screening of yeast strains that produce zero or low H_2S is particularly important for fermentation since there will be no sulfite formation during fermentation. The difference in H_2S production obtained in this study has been confirmed by several authors [16,27,28].

Concerning the growth of yeast strains at different temperatures, all strains had their optimal growth at 35°C, except the strains YD 7 and YD 21, which had their optimum growth at 30°C. This result is not surprising because [8] isolated yeasts from Cola cordifolia that grew optimally at 30 and 35°C. However, above 40°C, the growth rate drops considerably and becomes nil at 45°C. These results are similar to those of [29], who showed that the growth of strains isolated from cane juice becomes virtually impossible when the temperature reaches 40°C. According to [30], yeasts are stressed by high temperatures. They affect cell growth rate, ethanol production and cell viability [31,32].

Concerning the influence of pH, Isolate growth varies with pH. The isolates tested grew well between pH 4 and pH 7, reaching optimum growth at pH 4. These results are similar to those of [33], who showed that the growth of yeast strains from sweet potato and yam tubers remains maximal for pH values between 4 and 6.5. According to [34], yeast growth is favorable at acid pH.

For ethanol production, *Candida krusei* (YD33), *Candida guilliermondi* (YD37) and *Candida fermenti* (YD42) produced 6.23 ± 0.08% (w/v), 6.44 ± 0.07% (w/v) and 6.61 ± 0.01% (w/v) ethanol respectively in five days. Statistical analysis showed a significant difference between the quantities of ethanol prodiutes ($p < 0.050$). It should be noted that the ethanol production of these strains is similar to that of the ethanol production of yeast strains isolated from *cola cordifolia* by [8]. Indeed, ethanol production from yeasts isolated from this wild fruit ranged from 5.39 to 6.81%. In addition, the five best strains are non-*Saccharomyces* strains, which are known to modulate the aroma profile during alcoholic fermentation [35,36].

Isolates YD33, YD37 and YD42 showed the best characteristics (high CO2 production, no acetic acid production, high catalytic activity, high protease activity, low H2S production, and good resistance to temperature and pH variation) for fermentation. The growth of these yeast isolates under stress conditions confirms the ability of these yeast strains to be used as starters in many food processes, mainly in the fruit fermentation process.

5. Conclusion

Forty-six (46) pure isolates were isolated from *Diospyros mespiliformis* pulp. Among these Forty-six (46) yeasts isolates, three (3) isolates coded YD33, YD37 and YD42 showed the best characteristics, namely high CO_2 production, no acetic acid production, high catalytic and protease activity, low H2S production and good resistance to temperature and pH variation. These strains belong to three species: *Candida krusei* (YD33), *Candida guilliermondi* (YD37) and *Candida fermenti* (YD42). Thus, three (3) isolates with interesting technological properties could be used as starters in biotechnological applications.

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