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Production of ethanol and polyethanol by yeasts isolated from date (*Phoenix dactylifera L*.) wastes

Rezki Meriem Amina[1]*, Aouad Linda[2] and Bekki Abdelkader[1]

[1]Department of Biotechnology, Faculty of Nature and Life Sciences, University of Oran 1 Ahmed Ben Bella, Algeria.
[2]Department of Medicine, Faculty of Medicine, Djillali Liabes University, Algeria.

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The production of dates (*Phoenix dactylifera* L.) in Algeria generates each year, considerable quantities of waste that weaken the balance of our environment and are very rich in organic matter. Their valuation by biotechnological processes enables the production of high value added materials with low cost. In this regard, the objective of this study focused on the selection of yeasts that can be used to produce ethyl alcohol from this waste used in many industries and phenylethanol, an aroma popular in food, cosmetic and pharmaceutical. Among the three selected yeasts, RAM.20 generated up to 8 g/L of ethanol and excess of 800 mg/L of phenylethanol, and three sugars found in dates of the waste, namely glucose, fructose and sucrose. It is distinguished by its resistance to 18% ethanol (v/v) and 3 g/L phenylethanol. This strain naturally tolerates these products and is easy to handle and has a fundamental economic interest.

Key words: Yeasts, dates, waste, value, resistance, ethanol, phenylethanol.

INTRODUCTION

Dates (*Phoenix dactylifera* L.) have a very interesting nutritional value and are highly valued for their high concentration of antioxidants and their poverty in fat. In Algeria, the date palm is the backbone of agriculture in Sahara Africa by a very significant production (Kaidi and Touzi, 2001). This production and the various transformations result generate considerable quantities of waste that undermine the balance of our environment (Matallah, 1970; Bedrani and Benziouche, 2000). This waste, in addition to dates of low-value is very rich in organic matter and may comprise a raw material for many industries especially at this time where the needs of humans are becoming larger. It is therefore preferable to value them instead of destroying them in vain. Their valuation using the biotechnological processes represents a solution of choice to produce substances with high added value and low cost, contributing to the development of our industry and decreases some nuisance to the environment.

Among these substances, is ethyl alcohol, which is an

*Corresponding author. E-mail: rezkimeriem@yahoo.fr. Tel: 0550579755.

important energy base substance of numerous industries, such as chemicals, pharmaceuticals and cosmetics. The physicochemical properties compatible with gasoline also represent a promising alternative to fossil fuels; it is expensive and highly polluting (Maiorella et al., 1984; Bothast and Saha, 1997; Zaldivar et al., 2001; Farrell et al., 2006). As flavorings, depending on the market booms, its substances are often imported to meet consumer demands that are increasingly attentive to their health and the composition of their food. These consumers require aromas whose safety are guaranteed and that can be satisfied by the aromas produced microbiologically (Mayer, 1991; Chandrasekaran, 1997), especially by yeasts that have a very important metabolic potential. In this regard, this study was done to isolate yeasts from this waste and select the best candidate(s) for the production of these metabolites using sugars dates or glucose, fructose and sucrose.

MATERIALS AND METHODS

Isolation of yeasts and study of their characteristics

Yeast isolates were obtained from different samples of dates waste collected after processing of dates in Biskra in the Algerian Sahara. These isolates were isolated using the method of Ducastelle and Lenoir (1965). The collected waste was ground and homogenized in sterile mortars. One gram from the resulting paste of each sample is taken and dissolved in a sterile solution of sodium citrate 2% (w/v) previously heated to 45°C to soften, dissolve the constituents and to release microbial cells.

Each sample (0.1 ml) containing yeast is then plated on medium oxytetracycline glucose agar (OGA 0.5% w/v of yeast extract and 2% w/v glucose) with an acidic pH (5 to 5.6) and by the addition of two antibiotics (chloramphenicol 0.5 and 0.1 mg/ml of oxytetracycline) in order to delete a great number of bacteria (Ducastelle and Lenoir, 1965). The dishes were then incubated at 30°C. After microscopic examination, the yeast isolates were further purified by depletion method and stored on medium "sabouraud-chloramphenicol" (1% w/v of bactopeptone, 2% w/v glucose, 2% w/v agar and 0.5 mg/ml chloramphenicol) agar slant at 4 and -80°C on yeast extract-peptone-dextrose (YPD) medium supplemented with 25% v/v glycerol. During the final stage of purification, cultivation and morphological characteristics of these isolates were noted.

Media and culture conditions

The YP medium (1% w/v yeast extract, 1% w/v bactopeptone) to which sugar was added at a concentration of 2% (glucose (YPD), fructose (YPF) and sucrose (YPS)), was autoclaved at 120°C for 20 min.

The cultures were carried out in Erlenmeyer flasks containing one of the following media in the experiment: YPD, YPF and YPS from fresh subculture and incubated at a temperature of 30°C at 200 rpm in a rotary shaker. To assay the ethanol and phenylethanol (two metabolites on which this study was concentrated), samples were collected at various growth phases and assays were performed with supernatants using gas chromatography-flame ionization detector (GC-FID).

Determination methods

The analytical methods of chromatographic analysis by GC-FID for ethanol estimation are a column of RTX-BAC-2 (length: 30 m, diameter: 530 µm, thickness: 24 mm) where the injector temperature is 200°C, the detector at 250°C and the column at 40°C.

For phenylethanol, the analysis is performed by an Agilent apparatus 6890N and on a typical Agilent HP5 column (length: 30 m, diameter 320 µm, film thickness: 0.25 µm) under the following conditions: carrier gas: helium/injector: 250°C, split ratio = 10, volume injected = 2 µl/detector: flame ionization detector (FID), 270°C, flow H2 = 30 ml/min, air = 300 ml/min/thermal separation conditions (40°C/5 min)/ramp 30°C/min to 250°C.

Analysis of stressful conditions

Exposure to different stresses conditions was performed on cells in full exponential growth phase, with two duplicates in order to evaluate the performance of these isolates.

Five temperatures (25, 30, 37, 40 and 42°C) and a pH range (8, 7, 5, 4, 3 and 2.5) were tested using YPD medium. The same medium was then added to ethanol concentrations: 0 (T), 5, 10, 15, 18 and 20% (v/v) and phenylethanol 0 (T); 1; 2; 2.5 and 3 g/L.

A preculture of RAM.20 was prepared, centrifuged at 13000 rev/min for 3 min and washed with sterile distilled water. After a brief vortexing and centrifugation, the cells were resuspended with 100 µl of sterile distilled water. Sequential dilutions (10^{-1} and 10^{-2}) were prepared and 10 µl of each dilution was deposited on previously prepared sterile boxes. After drying, they were incubated at 30°C. The results was monitored and recorded daily for three days.

RESULTS AND DISCUSSION

Yeasts are widely distributed in nature and their ubiquitous appearance is once again confirmed by their presence in selected biological samples. From these habitats, isolation has led to the 12 isolates obtained, of which three were selected for this study. The results obtained show that the cultural characters of the three isolates' liquid and solid circles agree very well with those reported in the literature (Baffi et al., 2011). Regarding their morphology, the results of this study (Figure 1) show that it varies from one isolate to another. Indeed, it is spherical in MI.91, apiculate in MER.68 and ovoid in RAM.20. Their vegetative reproduction mode is by budding for all isolates. It is bipolar in MER.68 and multipolar in the other two. Note also that the presence of pseudomycelium, formed by a succession of elongated buds and branched chains, was observed in the latter.

Analysis of the production of ethanol and phenylethanol

Cell growth of these yeasts is coupled with the production of ethanol and other metabolites such as glycerol and acetates (not shown). Representing the maximum value of this output (Figure 2) allows RAM.20 to be distinguished

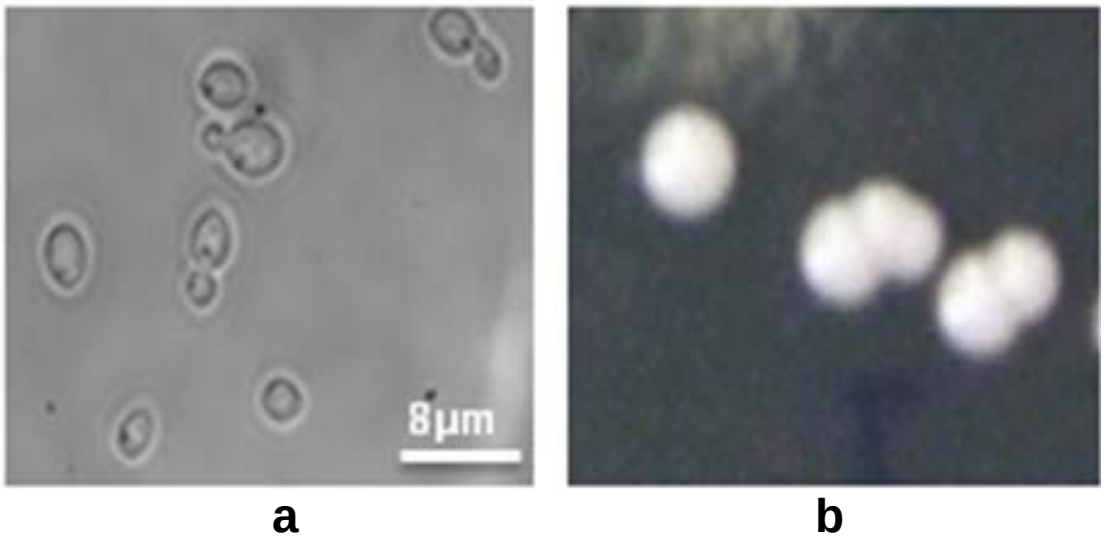

Figure 1. Macroscopic appearance (b) and microscopic appearance (a) on a young culture of RAM.20 on YPD medium/30°C.

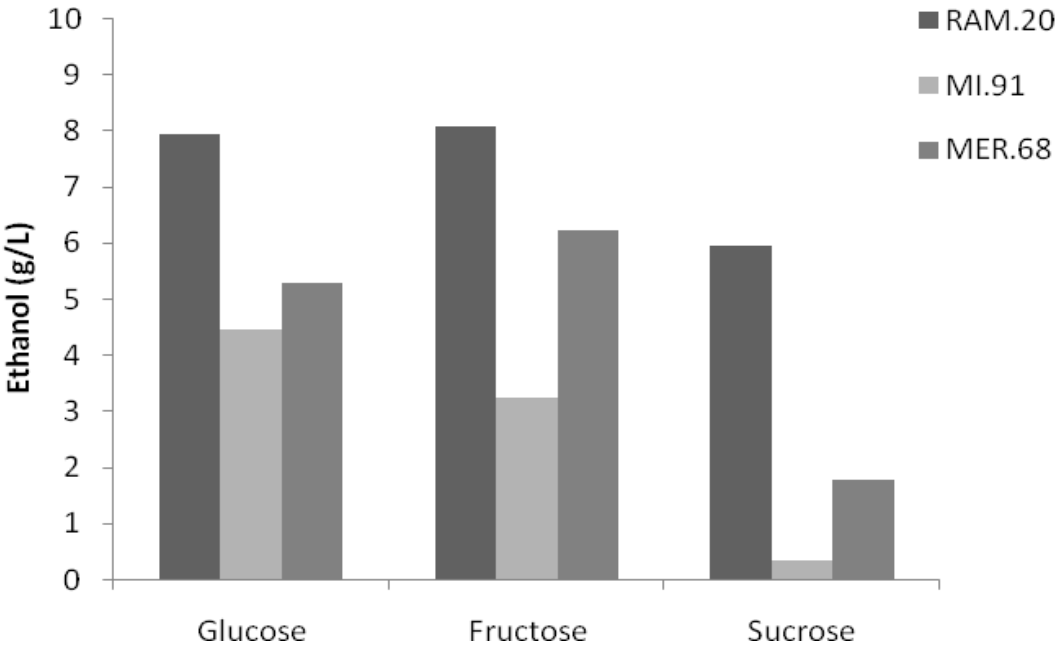

Figure 2. Production of ethanol by the three isolates (RAM.20, MI.91 and MER.68) using three glucidic substrates (glucose, fructose and sucrose) with the medium YP at a pH value of 6.5 and a temperature of incubation of 30°C/200 rpm.

which produces ethanol with three sugars giving a production of around 8 g/L with the glucose and fructose and 6 g/L with sucrose, unlike the other two isolates (MI.91 and MER.68) whose production is lower especially with sucrose. Indeed, several studies have confirmed the assimilation of these two sugars (glucose and fructose) in a very large number of yeast species (Barnett et al., 2000; Basilio et al., 2008; Gallardo et al., 2010).

An insignificant phenylethanol production is observed in MI.91. The production of MER.68 is between 400 and

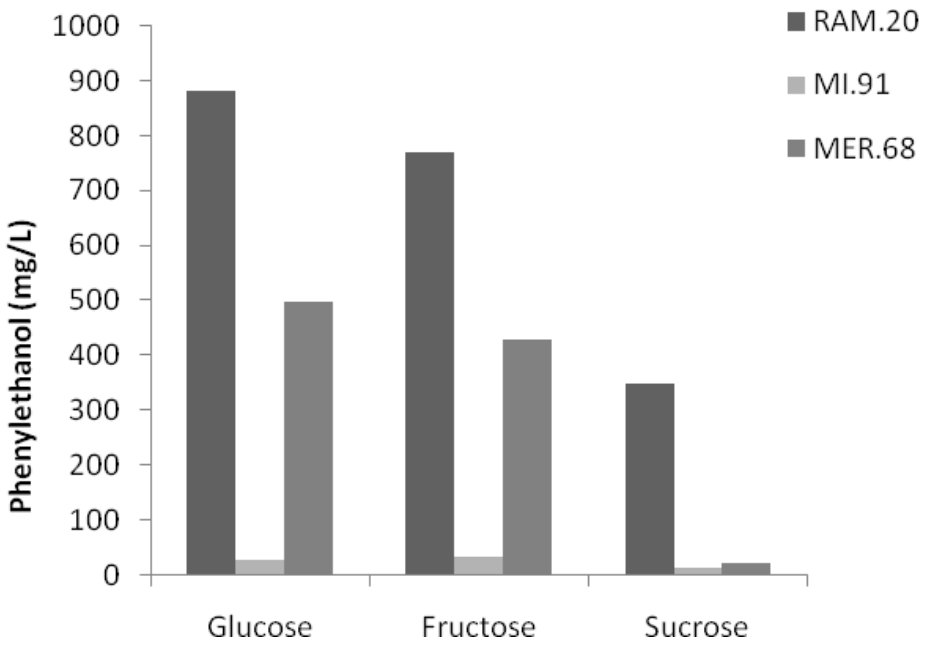

Figure 3. Production of phenylethanol by the three isolates (RAM.20, MI.91 and MER.68) using three glucidic substrates (glucose, fructose and sucrose) with the medium YP at a pH value of 6.5 and a temperature of incubation of 30°C/200 rpm.

Table 1. Effect of temperature on the three isolates of yeasts studied with the medium YP at a 30°C temperature of incubation.

Isolate	25°C	30°C	37°C	40°C	42°C
RAM.20	+	+	+	+	+
MI.91	+	+	+	+	-
MER.68	+	+	+	+	-

500 mg/L, but with the glucose and fructose only. In contrast, RAM.20 is distinguished by a high production with three carbohydrate substrates, particularly, glucose where it exceeds 800 mg/L (Figure 3). The production of this higher alcohol in a rich medium such as YPD is the result of the bioconversion of excess branched chains and amino acids present in the culture medium by the catabolic pathway Ehrlich (Hazelwood et al., 2008).
This isolate (RAM.20) thus stands out for its significant production capacity to ethanol and phenylethanol with three sugars (glucose, fructose and sucrose) contained in the waste dates.

Analysis of tolerance to stressful conditions

These stress conditions, regarded as variations in the yeast culture medium, impact their behavior by affecting their growth potential and their production, causing a malfunction consequently up to the death of these cells (Estruch, 2000; Hohmann, 2002).

Effect of temperature

Knowing that the temperature has a direct effect on cell viability and production of ethanol (Aldiguier et al., 2004), its impact on selected yeast isolates was studied and the results show that MI.91 and MER.68 are unable to maintain their growth beyond 40°C. Against the growth of RAM.20 isolate which is affected above 42°C (Table 1). These results are in agreement with the heat-tolerant properties of certain yeast species studied by several authors (Gallardo et al., 2010; Gallardo et al., 2011; Kwon et al., 2011; Yuangsaard et al., 2013). These authors attribute this resistance to their plasma membrane which is resistant to thermal denaturation. Our results show that due to its resistance to high temperatures,

Table 2. Effect of pH on the three isolates of yeasts studied with the medium YP at a 30°C temperature of incubation.

Isolate	8	7	5	4	3	2.5
RAM.20	+	+	+	+	+	+
MI.91	+	+	+	+	+	-
MER.68	+	+	+	+	+	-

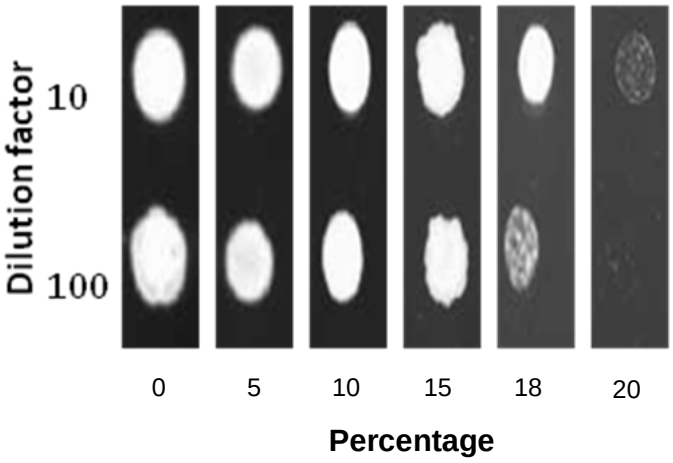

Figure 4. Growth of RAM.20 at different concentrations of ethanol (v/v) on solid YPD incubated at 30°C.

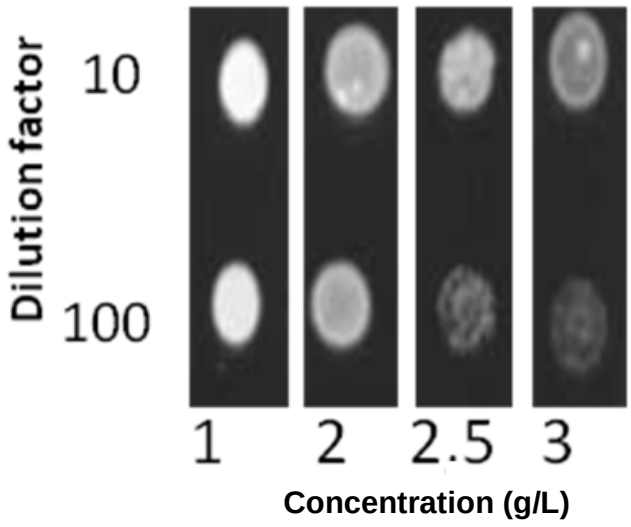

Figure 5. Growth of RAM.20 at different concentrations of phenylethanol (g/L) on solid YPD incubated at 30°C.

RAM.20 isolate may therefore be of great value for certain biotechnological applications especially when the temperature exceeds 39°C in a continuous fermentation system.

Effect of pH

The pH effect was determined for the three isolates, in particular, the effect of acidic pH since the acidity is known for its inhibitory effect on the growth of yeasts (Thomas et al., 2002), slowing the consumption of sugar and hence reducing the productivity (Torija et al., 2003). It can also affect active nitrogen transport (Dubois, 1979; Grenson, 1979; Gregory et al., 1982) important for the growth of yeast. The results obtained show that the three isolates can tolerate a 3 pH value, however the RAM.20 continues to grow at pH up to 2.5 (Table 2).

Effect of ethanol

The impact of ethanol, a major stress factor which diffuses through the plasma membrane, inhibits growth (Piper, 1995), decreases the viability of these cells (Bai et al., 2004), greatly affects the functions and the physicochemical properties of their plasma membrane (Alexandre et al., 1994; Alexandre et al., 1998), and thus reduces the ethanol yield (Pina et al., 2004), and has been studied only in yeast RAM.20 to give an interesting ethanol production with three sugars (glucose, fructose and sucrose), and the growth showed no drop. The result, shown in Figure 4, shows that RAM.20 is very resistant to the toxicity of ethanol and significantly since it can grow in the presence of 18% (v/v) ethanol. Different strategies have been used to improve ethanol production and cell viability and failure in creating a yeast strain with much improved tolerance, naturally resistant to ethanol yeasts can result in fundamental scientific and economic interest for manufacturers.

Effect of phenylethanol

Similarly to phenylethanol that is used in various fields and is in second place after ethanol commercially, whose sensitivity is a serious problem and has prompted researchers and industry to adopt very expensive methods, such as extraction of the flavoring. Naturally resistant strains are preferred because they have some advantages over the cost and handling and this yeast is resistant to 3 g/L phenylethanol (Figure 5).

These results show that RAM.20 is multi-drug resistant and the ability to tolerate various stress is one of the

important criteria in the selection of strains for the efficient fermentation. It can be an ideal candidate for the valuation dates of waste by producing ethanol and phenylethanol and for many who are economically very interesting. This enhancement also allows users to reduce pollution by the wastes that represent a real problem in the Sahara bracket.

Conclusion

The yeasts studied are wild strains from a particular biotope unexplored and poorly studied. The study of their physiology that focused on the assimilation of three carbon substrates found in the waste of dates indicates the importance of RAM.20 yeast in the production of ethanol with values ranging between 6 and 8 g/L and the production of phenylethanol which can reach 900 mg/L. The isolate RAM.20 stands out by its high production of both metabolites and its high tolerance to some stress. It is resistant to a temperature up to 42°C, high acid pH of 2.5, 18% ethanol (v/v) and 3 g/L of phenylethanol. This is what has shown the importance of this yeast that has very interesting potential in terms of strength and shows significant interests in biotechnology, mainly in the production of bioethanol and phenylethanol.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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