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SCREENING OF POTENTIAL YEAST STARTERS WITH HIGH ETHANOL PRODUCTION FOR A SMALL-SCALE COCOA FERMENTATION IN IVORY COAST

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Abstract: *Cocoa beans (Theobroma cacao L.) are the raw material for chocolate production. The variability of cocoa beans quality, due to the activity of microbiota involved in cocoa fermentation causes huge economic losses in cocoa-producing countries. To resolve this issue, 743 yeast strains isolated in Ivorian cocoa fermentation were investigated in order to identify potential starters by their ability to resist to physico-chemical stress conditions and to produce enzyme necessary to cocoa fermentation. Thus, out of the 743 investigated yeast strains, 113 showed high fermentative capacity, 19 being able to produce high ethanol contents up to 4,18 % w/v. Out of 19 yeast strains high ethanol producers, two strains Saccharomyces cerevisiae YB14 and Pichia kudriavzevii YP13 were able on one hand to resist to most parameters such as temperature, pH, ethanol, organics acid, and on the other hand they were able to produce specific enzymes like pectinase, β-glucosidase, protease necessary to have a good cocoa fermentation process, and finally to produce acetoin which is desirable for flavor development in the fermentation process. These two strains could therefore be used as starter cultures which may contribute to the control of cocoa fermentation in Ivory Coast.*

Keywords: Yeasts, ethanol, cocoa fermentation, starters

1. Introduction

Cocoa fermentation plays an important role in the chocolate production chain with major impacts on product quality and value [1]. It's a spontaneous process which involves the action of complex microbial interactions, led mainly by yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and aerobic spore forming bacilli (*Bacillus sp.*). The microbial activity generates metabolites and conditions that kill the embryo of beans, triggering an array of biochemical reactions, essentials for cocoa quality development [2, 3]. Among microflora of cocoa, yeasts are frequently isolated from fermenting cocoa beans. They perform mainly an alcoholic fermentation of pulp sugars to produce ethanol and carbon dioxide, and secondary metabolites, such as higher alcohols, organic acids, esters, aldehydes, ketones, sulfur, and nitrogen volatiles, which will diffuse into the bean to affect the final quality of chocolate [4, 5]. Moreover, others functions such as acceleration of carbohydrate consumption, pectinolytic activity and inhibition of growth of pathogenic microorganisms, essential to cocoa fermentation are also attributed to yeast metabolism [6, 7]. Regarding all these aspects, the use of yeast starter cultures has been proposed as one the key to conduct controled cocoa fermentations. In this context, some species including *Saccharomyces cerevisiae*, *Kluyveromyces*

marxianus, *Torulaspora delbrueckii*, *Pichia kluyveri*, *Hanseniaspora uvarum* and *Candida sp.* were used as starter, and the results obtained contributed to successful changes in cocoa aroma profile with respect to spontaneous cocoa fermentation [7-11].

Although, these different species improved cocoa fermentation process and the chocolate quality, the results obtained are still insufficient for cocoa fermentation process standardization. Therefore, studies to select appropriate yeast starter have to be pursue in order to solve the great problem of the variability of cocoa beans quality linked to the spontaneous nature of fermentative cocoa process [12]. In this context, the use of yeast starters with high ethanol production capacity was suggested. In fact, ethanol plays an important role in cocoa fermentation. It contributes to the brown color of good fermented cocoa beans; because it facilitates glycosidase and polyphenoloxidase action, responsible to color change [13-15]. Through its oxidation in acetic acid, ethanol contributes highly to characteristics aromas formation of chocolate [16, 17]. Indeed, during cocoa fermentation, ethanol produced mainly by yeasts, is oxidized into acetic acid by acetic acid bacteria. The ethanol oxidation in acetic acid increases cocoa mass temperature which kill the cocoa-beans embryo. The death of cocoa beans allows the permeability of cellular-wall, which facilitate the diffusion of metabolites (ethanol, acetic acid) within cotyledons. This diffusion activates reserve-cellules enzymes, which produce cocoa aromas precursors [18-20]. More to the point, the studies of [11] showed that the addition of yeasts starter sole at the beginning of fermentation contributes to have higher AABs concentration in mass fermenting and a better cocoa beans quality comparatively to spontaneous fermentation. In addition, according to [21], a high acetic acid amount would contribute to obtain a good cocoa-beans quality. Therefore, this high acetic acid amount during the fermentative process could come from high ethanol amount from yeasts activity. Thus, yeasts with high ethanol production capacity may be useful for cocoa fermentation. This would allow AABs to have the early ethanol for conversion to flavor precursors, but also could contribute to the reduction of fermentation time.

So, the aim of this study is to select potential yeast starters with high ethanol production capacity for the control of cocoa fermentation. This study, considered as a promising approach, is the first which pointed exceptionally the effects of stress conditions from cocoa fermentation on ethanol production for the selection of yeast starters.

2. Materials and methods

2.1. Yeast strains isolation

A total of 743 yeast strains were previously isolated from six cocoa fermentations carried out in Ivory Coast. Theses strains were stored in MYGP broth supplemented with 20 % glycerol for further tests [12].

2.2. Screening of potentials yeast starters

2.2.1. Screening of high fermentative-capacity yeasts

The fermentative capacity of 743 yeast strains was studied according to the method of [22] with a slight modification. From pre-culture of 24 hours, pure yeasts culture were suspended in saline tryptone to get an optical density of 0.7 at 600 nm and 100 µL of this suspension were used to inoculate 10 mL of YPG medium containing a Durham tube into essay tube.

Then, the culture was incubated at 30 °C for 6 days, without agitation. Fermentative capacity was also determined by measuring the gas production in Durham test. Yeasts usually oxidize sugars into ethanol with production of gas [23]. This volume of gas and ethanol produced is related to fermentative strength of strain [24].

2.2.2. Screening of high ethanol-producer yeasts

The screening was carried out by the method described by [25] with a slight modification. From pre-culture of 24 h, pure yeasts culture was suspended in saline solution buffer to have an optical density of 0.7 at 600 nm. 100 µL of this suspension were used to inoculate 10 mL of YPG medium. Then, the culture was incubated at 30 °C for 3 days, without agitation. After 72 h, the culture was centrifuged at 15000 rpm for 10 min at 4 °C and the supernatant was retained. The supernatant containing ethanol produced by yeasts is quantified by UV Method using alcohol dehydrogenase (ADH) and NAD as cofactor. Thus, the produced ethanol is measured by following the formation of NADH to 340 nm at spectrophotometer (*Eppendorf*, Germany) according to the reaction:

$$RCH_2OH + NAD^+ \xrightarrow{ADH} RCHO + NADH + H^+$$

The reactional medium is composed of 0.75 mL of H_2O; 0.25 mL of buffer 60 mM Tris-HCl (pH 9); 0.05 mL of 1.5 mM NAD^+ (Sigma); 1µL of ADH (500U/mL, Sigma) and 50 µL of the supernatant to be analyzed. The ethanol production was determined using a standard curve carried out under the same conditions as the test with 99 % pure ethanol.

2.3. Effects of cocoa fermentation environmental conditions on ethanol production by yeasts

The yeasts with high ethanol production capacity were further investigated to assess the individual impact of various environmental conditions (notably pH and temperature) and organic compounds (ethanol, citric acid, lactic acid and acetic acid) on ethanol production according to the method of [25] with slight modifications.

2.3.1. Ethanol production under temperature and pH stress

Strains were grown in a 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 ethanol production by yeasts, 10 mL of standard liquid medium contained in a 50 mL test tube, were inoculated with 100 µL of yeasts pre-culture, OD_{600} = 0.7. Then, cultures were incubated for 72 h at variable temperatures ranging from 30 to 45 °C. Influence of pH variations on ethanol production were analyzed in the same medium at different pH values (3; 4 and 5), and incubated at 30 °C.The ethanol production was quantified by the UV Method using alcohol dehydrogenase (ADH) and NAD.

2.3.2. Ethanol production under carbohydrates stress

The influence of sugars (glucose, fructose and sucrose) on ethanol production by yeasts was also individually assessed. The liquid medium contained 0.05 % yeast extract; 0.3 % casein peptone with sugar at different concentrations. The different sugar concentrations studied were 2; 4 and 8 % for glucose and fructose, and for sucrose 2; 4 and 6 %. After inoculation, each medium was incubated at 30 °C during 3 days.The ethanol production was

quantified by the UV Method using alcohol dehydrogenase (ADH) and NAD.

2.3.3. Ethanol production under ethanol and organic acidsstress

The influence of each organic compound (ethanol, citric acid, lactic acid and acetic acid) on ethanol production by yeasts was individually assessed. For this purpose, the standard liquid medium free of these compounds was used as negative control. For example, to assess the influence of ethanol on ethanol production, yeasts were grown in the standard medium containing variable ethanol concentrations (4; 8 and 12 %), incubated at 30 °C and then ethanol production in these variable ethanol concentrations was compared with the negative control. The same type of experiment was performed individually for each compound at variable concentrations of acetic acid (0.5; 1 and 2 %), lactic acid (0.5 and 1 %) and citric acid (1; 2 and 3 %). The ethanol production was quantified by the UV Method using alcohol dehydrogenase (ADH) and NAD.

2.4. Enzymatic activities and acetoin production of high ethanol-producer yeasts

2.4.1. Enzymatic activities

The yeast strains were analyzed for enzymatic activities, and in particular for the esterase, β-glucosidase, glycosidase, protease and pectinase activities, according to the methods reported in [26]. Screening of enzymatic activity was performed in triplicate on agar plates by spot inoculation method. The positive yeast controls, used as the reference in order to verify the reliability of the tests, were part of the collection of the laboratory of Biotechnology of Felix Houphouet-Boigny University.

2.4.2. Acetoin production

Acetoin production was demonstrated according to the method described by [27]**.** The Clark and Lubs medium is inoculated with yeast strains and incubated at 30 °C for 48 h. After incubation, 1 mL of the medium is taken and introduced in a test tube. To this medium are added two drops of an alcoholic solution of 6 % alpha-naphthol in 90 ° alcohol and two drops of a 16 % sodium hydroxide solution in distilled water. The tube tests are stirred and heated on the flame of a Bunsen beginning of boiling. After stirring for 30 seconds on a vibrating stirrer of vortex type, a cherry red color indicates a positive reaction.

2.5. Phenotypic and molecular characterization of potential starters

2.5.1. Phenotypic characterization of potential starters

The best high ethanol-producer yeasts were investigated for growth at different culture conditions according to the method described by [28]. From pre-culture of 24 h, pure yeasts culture was suspended in saline solution buffer to have an optical density of 0.7 at 600 nm. 100 µL of this suspension were used to inoculate 10 mL of YPG medium in 50 mL test tube. The strains were tested for growth in YPG at different pH (3; 4 and 5) and incubated 30 °C with 150 rpm orbital shaking during 3 days. The growth was also assessed in YPG (natural pH 5.6) at different temperatures 30; 35; 40; 45 and 50 °C with 150 rpm orbital shaking during 3 days. After incubation time, the growth was determined by measuring the optical density at 600 nm at spectrophotometer (*Pioway medical Labs*, Singapore).

Different sugars were tested using a base of yeast extract (0.05 %) and peptone (0.3 %) additioned of glucose (2; 4 and 8 %), fructose (2; 4 and 8 %) and sucrose (2; 4

and 6 %). Furthermore, growth in presence of ethanol, lactic acid, citric acid and acetic acid was tested. The different concentrations were tested for ethanol (4; 8; 12 and 16 %), citric acid (1; 2; 3 %), acetic acid (0.5; 1; 2 %), lactic acid (0.5; 1%) and supplemented by YPG.

The high osmotic tolerance test was carried out according to [29] in two steps. Firstly, a basal medium containing 0.5 % yeast extract and 0.5 % peptone was supplemented with 50 % of glucose to determine the ability to growth yeast at high level sugar [30]. Secondly, a medium containing 10 % sodium chloride and 5% glucose [31] was realized to determine the ability to resist at high osmotic pressure. For both experiments, the growth was assessed by measuring the optical density at 600 nm after 3 days of incubation at 30 °C with shaking at 150 rpm.

2.5.2. Molecular characterization of potential starters

Yeasts were grown at 30 °C to the mid-log phase in YPD medium before harvesting. Yeast genomic DNA was extracted using the classic phenol / chloroform method described by [32]. The D1/D2 region of 26S rDNA was PCR amplified as described by [33]using the eukaryotic universal primer gc-NL1 (5' CGCCCGCCGCGCGCGGCGGGCGGGC GGGGGCCATATCAATAGCGGAGGA AAAG 3') and the reverse primer LS2 (5' ATTCCCAAACAACTCGACTC 3'). The D1/D2 PCR program consisted to one cycle at 94 °C for 3 min, followed by 30 cycles (95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min), and a final extension at 72 °C for 10 min. PCR-amplified D1/D2 regions of 26S rDNA were sequenced by BIOFIDAL (Lyon, France) using the Pseq D1/D2 primer (5'GGGCCATATCAATAAGC3'). Sequences were then compared to the NCBI data Genbank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences showing a high percentage of identity (≥ 97 %) were considered as belonging to the same species.

3. Results and discussion

3.1. Fermentative capacity and ethanol production of yeast strains

Among the 743 analyzed yeast strains for the fermentative capacity, only 113 showed a high fermentative capacity with gas amount above to 4 cm^3 (table 1).

Table 1.
CO_2 production of yeast strains involved into cocoa fermentation in Ivory Coast.

Fermentative capacity	Volume of CO_2	Number of isolates
Low level	[0 ; 2 cm^3 [	506
Middle level	[2 ; 4 cm^3 [	124
High level	[4 ; 6 cm^3]	113

These yeast strains are susceptible to product high level ethanol because during ethanol fermentation, gas produced is correlated to ethanol produced according to [24]. Indeed, fermentations that develop a stronger presence of highly fermentative yeasts are more likely to have a greater concentration of ethanol [34-36] than those which develop less-fermentative yeasts [37].

Hence, the 113 yeast strains with a high fermentative capacity strains were investigated to select among them, the high ethanol-producer yeasts. The results indicated that 62 (54.87 %) yeast strains have ethanol production inferior to 1 % (w/v) while 32 (28.32 %) yeast strains have ethanol production within 1-2 % (w/v) and 19 (16.81 %) yeasts strains produce ethanol above 2 %. The table 2

shows the distribution of ethanol production level for the 19 yeasts strains high ethanol-producer.

Table 2.
Ethanol production by yeast strains with high fermentative capacity
Each data is the average of triplicate tests analysis.

Yeast strains	Ethanol production (% w/v)
YP20	4.19 ± 0.16^{a}
YT13	3.23 ± 0.03^{b}
YT8	3.09 ± 0.05^{bc}
YP11	2.77 ± 0.07^{cd}
YG14	2.69 ± 0.09^{de}
YP9	2.58 ± 0.10^{def}
YH7	2.49 ± 0.20^{def}
YT11	2.37 ± 0.29^{efg}
YP3	2.31 ± 0.00^{efg}
YG28	2.28 ± 0.00^{fg}
YP23	2.27 ± 0.20^{fg}
YB11	2.19 ± 0.01^{fgh}
YB10	2.08 ± 0.03^{ghi}
YP13	2.07 ± 0.00^{ghi}
YP12	2.05 ± 0.01^{ghi}
YB14	1.79 ± 0.16^{i}
YC4	1.78 ± 0.10^{i}
YG12	1.78 ± 0.24^{hi}
YP1	1.72 ± 0.22^{i}

Data following with the same letter were similar statistically at 95 % according to Tukey test.

During most cocoa heap fermentation, the ethanol concentration into pulp doesn't exceed 20 mg/g cocoa beans whether 2 % (w/v) during the first 24-72 h [6, 9, 35, 38-41]. However, according to [42] and [43], ethanol concentrations in excess of 40 mg/g (4 %) are needed to kill the bean, important step into cocoa fermentation. Indeed, the death of bean due to ethanol and acetic acid diffusion will trigger endogenous biochemical reactions that produce the chocolate flavor precursors [2, 44, 45]. These lasted will be transformed into distinct chocolate notes during the subsequent industrial processes [46].
Ethanol produced by yeasts, could contribute also into brown color obtain of cocoa beans fermented, as will facilitate the bean glycosidase activity necessary to convert the bean anthocyanins (purple-red) to the colorless form [13, 14] and the bean polyphenoloxidase activity needed to transform phenolic compounds to brown polymers [15].
Thus, the nineteen (19) yeast strains able to produce ethanol more than 2 % (w/w) will contribute to have ethanol concentrations in excess of 40 mg/g (4 %), which, are needed to kill the bean in order to reduce the fermentation time and obtain a good cocoa beans fermented.
However, during cocoa fermentation, the environmental stresses impact the microflora activity, which influence the efficiency of cocoa fermentation. The impact of stress conditions prevailing during cocoa fermentation process on the ability of these 19 yeast strains to produce ethanol must be study for an efficiency selection of appropriate starters.

3.2. Effect of pH and temperature on the ethanol production

The influence of temperature on yeasts for ethanol production indicates that all the 19 yeast strains are able to produce ethanol at 30; 35 and 40 °C. In addition, the maximal productions were recorded to 30 – 35 °C for most strains. Nevertheless beyond 40 °C, an inhibition of fermentative yeasts activity was observed (figure 1).
These results showed that the increasing temperature influences negatively the fermentative activity of yeasts. Indeed, temperature is the main environmental parameter which has an important impact on physiology and activity of microorganisms [47]. It affects growth rate, speed production, CO_2 production, ethanol production and cellular viability [48]. Also, according to [49] and [48], beyond 35 °C it appears to most yeasts a real decreasing of ethanol rate production. However, in our study, twelve (12) yeast

strains showed a good ethanol production at 35 °C and these strains kept 50 % of their ethanol production at 40 °C comparatively to 30 °C.

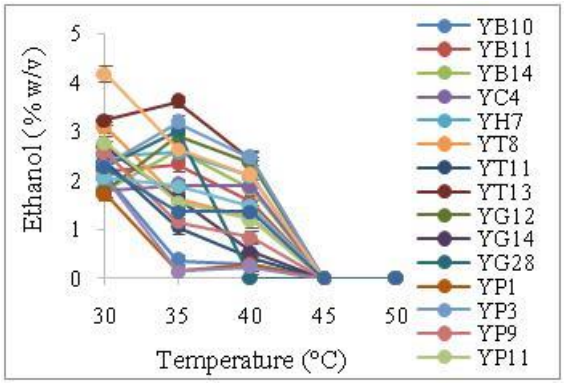

Fig. 1. Effect of temperature on ethanol production of yeasts

The influence of pH on ethanol production indicates that these twelve (12) yeast strains, able to produce ethanol with ethanol production capacity under high temperature, are also able to produce ethanol at pH 3, pH 4 and pH 5 (figure 2).

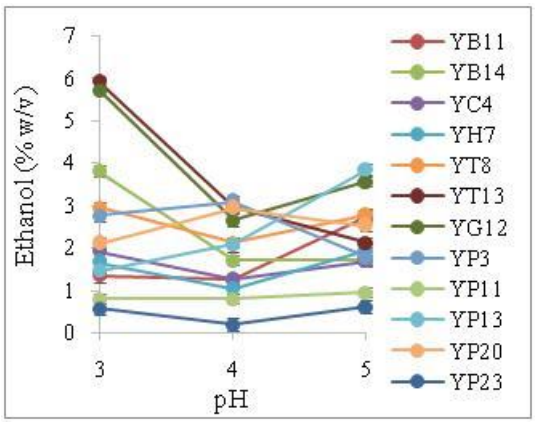

Fig. 2. Effect of pH on ethanol production of yeasts

However, the high levels of ethanol production were recorded for pH 3 and pH 4. Among these twelve yeast strains, seven (7) strains (YP3, YP13, YP20, YB14, YT8, YT13 and YG12) showed high production levels at pH 3 and pH 4 comparatively to the other yeasts; and the ethanol production for these strains ranged to 1.70 – 3.84 % (w/v) for pH 3 and 1.69 – 3.07 % (w/v) for pH 4.

At the beginning of cocoa fermentation, the cocoa pulp has acid pH (3-4) which constitute favorable conditions to yeasts growth [20].

Thus, these seven (7) yeast strains, which are able to produce high ethanol rate at 35 – 40 °C and at pH 3 and pH 4, could be able to start the cocoa fermentative process but will also be able to produce ethanol during the first 48 – 72 h when temperature reach 35 – 40 °C [20].

However, pH and temperature are not sole stress conditions which prevailing into cocoa fermentation. Variations of sugar, ethanol and organic acid concentrations influence strongly the yeasts activity. It arranges to study the effect of these stresses on ethanol production by yeasts for selecting appropriate starter cultures.

3.3. Effect of carbohydrates and organic compounds on the ethanol production

The figure 3 (A, B, C) showed that all the seven (7) yeast strains realized an alcohol fermentation with glucose and fructose until 8 %, and sucrose until 6 %. However, the ethanol production differs to one yeast strain to another for each sugar.

The results indicate also that all yeast strains excepting YT8 showed ethanol production more than 2 % with different sugars studied. The ethanol production by these yeast strains confirms the known role of yeasts, to degrade sugars into ethanol as reported by literature data [29, 50]. Thus, the mucilaginous cocoa bean rich-pulp in fermentable sugars as glucose, 2.4–6.6 % (w/w), fructose 4.2–7.4 % (w/w), and sucrose, 2.1–3.2 % (w/w) [16, 34, 37, 51, 52] would constitute favorable conditions for fermentative activity of these yeast

strains during fermentation process of beans.

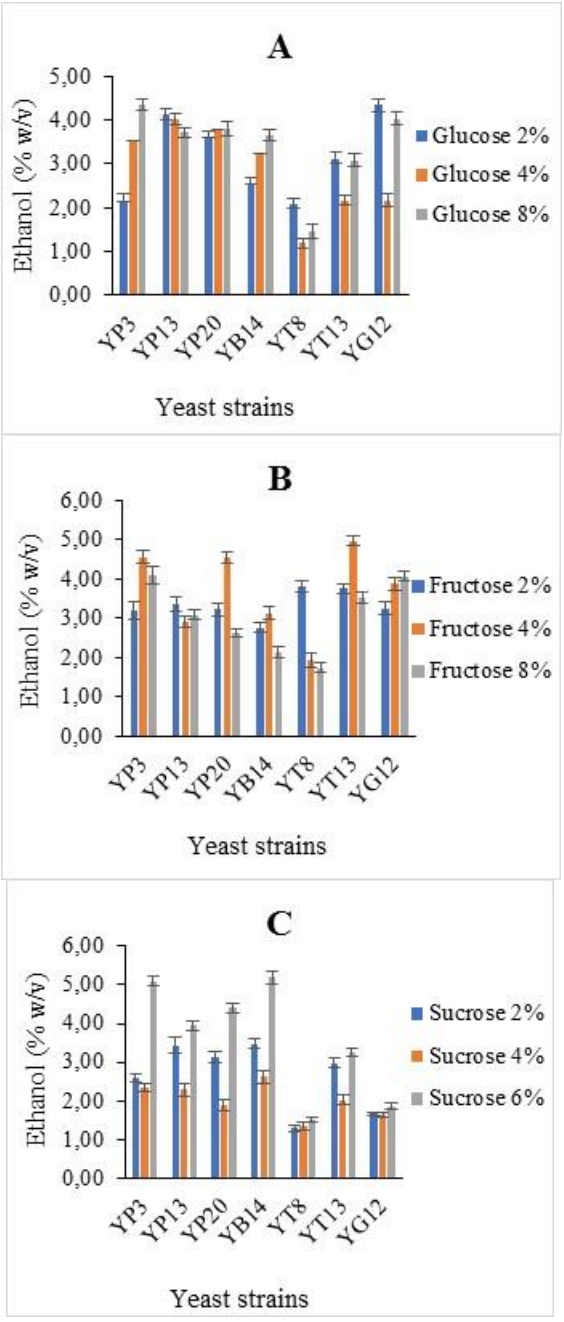

Fig. 3. Effect of glucose (A), fructose (B) and sucrose (C) on ethanol production of yeasts

So, the seven yeast strains tested excepted YT8 would be susceptible to realize easily alcohol fermentation during the cocoa fermentative process to produce ethanol necessary at good fermentation.

The figure 4A showed that ethanol production decreases when ethanol concentration increases until to have an inhibition in presence of ethanol 8 % in the medium.

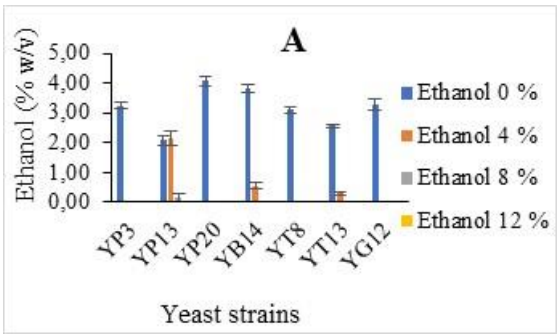

Fig. 4A. Effect of ethanol concentration on ethanol production of yeasts

This indicates that ethanol is able to limit the ethanol production itself as reported by some authors [53-55]. Indeed, according to these authors, when ethanol concentration increases into the culture medium, a decrease of rate growth, cellular viability, metabolic activity and production ability is observed. However, some yeast strains as YP13, YB14 and YT13 showed a resistance to ethanol indicated by their ethanol production at presence of ethanol 4 % in the medium. Among these yeast strains, YP13 showed good ethanol production in presence of 4 % ethanol in the medium compared toYB14 and YT13. A lower production in presence of ethanol 8 % in the medium is recorded by YP3 while ethanol production by YB14 and YT13 were inhibited. These results corroborated those of [56] and [57] which reported a threshold and maximum tolerance level 4 % (w/v) and 8 % (w/v) ethanol for yeasts, respectively. The maximum concentrations of ethanol reported in the pulp and nib fractions vary from as little as 0.5–2 % [6, 35, 37, 58] to

values as high as 3–8 % [28, 34, 36, 59]. Although, our yeast strains can't produce ethanol above ethanol 8%, they may be relevant to cocoa fermentation because the high ethanol concentrations produced by yeasts during the first 48-72 h to cocoa fermentation time, could rapidly convert by acid acetic bacteria (AAB) which are dominating in this time. Indeed, ethanol produced by yeasts would be oxidized into acetic acid by AAB, which will diffuse into cotyledons, for formation of aroma precursors future chocolate [20, 46, 60].

Citric, acetic and lactic acids have key roles in the development of chocolate characterics [44, 59, 61]. Apart from their direct impacts on sensory quality, their diffusion in and out of the nib modulates bean pH which indirectly affects the activity of bean enzymes such as proteases and invertase involved in the production of free amino acids, peptides and reducing sugars [17, 44, 62]. They have an inhibitor effect on the growth and activity of fermentative microbiota notably yeasts; due to their un-dissociated forms [63]. Indeed, in acid medium, the un-dissociated acids can diffuse through plasmic membrane, in intracellular environment more alkaline (cytoplasm) where the dissociation happens [64-66]. These dissociated acids disarrange cellular activity employing for example an uncoupling effect on respiration or inhibition on some enzymes activities notably the obstruction of some transport mechanisms, which can cause the loss of cellular activity then cellular death.

The results of this study indicate that citric acid, main acid in unfermented cocoa pulp, influences fermentative activity of yeasts (figure 4B). Out of the 7 yeast strains tested, YP3, YP13, YB14 and YT8 showed ethanol production inferior to 1 % (w/v) in presence of citric acid 1 % (w/v) in the medium. However, beyond 1 % citric acid, it was observed an inhibition of fermentative activity for all the 7 yeast strains.

This inhibition activity of yeast strains would confirm that the citric acid use is more often connected with the growth of certain species of lactic acid bacteria, which are present at the first hours of the fermentation time with yeasts, such as *L. plantarum* as reported by [67] and [68] or *Lactococcus lactis* and *L. fermentum* as reported by [16].

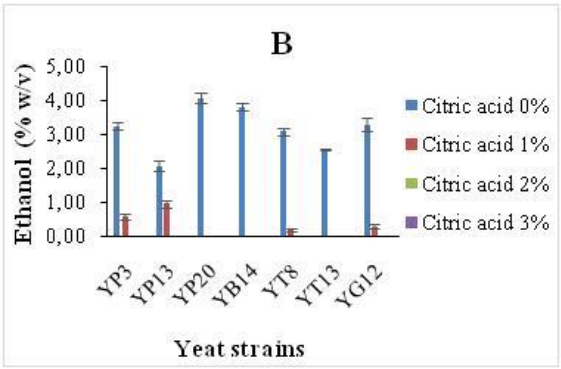

Fig. 4B. Effect of citric acid concentration on ethanol production of yeasts

During cocoa fermentation, the citric acid in pulp is variable depending on cocoa pods source, probably due to different varieties of cocoa pods which exist in the world. In Indonesia, cocoa pulp contains 2.1-2.4 % (w/w) citric acid while in West Africa notably in Ivory Coast, cocoa pulp is around 1 % citric acid [13, 34, 38, 69]. Thus, YP3, YP13, YB14 and YT8 could be able to start cocoa fermentative in Ivory Coast with a high ethanol production under conditions where acid citric concentration don't exceed 1% citric acid.

Concerning acetic acid, the figure 4C indicates that acetic acid decreases fermentative activity of yeast strains. Indeed, rapid fall on fermentative activity was observed between 0-0.5 % acetic acid (figure 4C).

These results corroborate those of [70] who reported that no ethanol was produced when 0.5 % (w/v) acetic acid was present in brewing mash. Moreover, according to [71], acetic acid in excess of 0.1 % (w/v) decreased the rate of ethanol production. However, YP13, YT8, YT13 and YG12 showed ethanol production counting to 0.11 – 0.53 % (w/v) in presence of acetic acid 0.5 %.

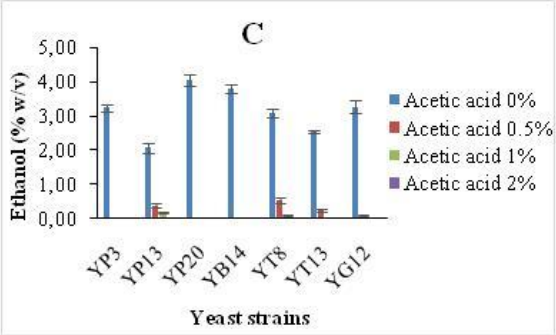

Fig. 4C. Effect of acetic acid concentration on ethanol production of yeasts

Among these yeast strains, YP13 and YT8 produce lesser ethanol values around 0.1 % (w/v) with 1 % acetic acid in medium. During the first hours of Ivorian, Malaysian and Ghanaian cocoa heap fermentation, the acetic acid concentrations counting 0.6 – 1 % [38, 67, 69]. Moreover, the production of acetic acid at levels of 1–2 % during fermentation is necessary to kill the bean [43] and it is generally considered that this production mainly comes from the oxidation of ethanol by acetic acid bacteria [2, 17, 44]. Despite their low production recorded at 1 % acetic acid, YP13 and YT8 would be able to resist to acetic acid stress conditions encountered during cocoa fermentative process. However, the high level of ethanol production by these yeasts at the beginning of cocoa fermentation, when acetic acid content is 0.04 % [34, 36], could allow AAB to produce the acetic acid necessary to kill the bean and trigger endogenous biochemical reactions for the formation of chocolate flavor precursors [2, 16, 45].

As for lactic acid, the figure 4D indicates that lactic acid inhibits highly fermentative yeasts activity. Indeed, the yeast strains cannot produce ethanol to 0.5 and 1 % lactic acid.

These results corroborate those of [72] which reported that lesser concentrations of 0.2-0.6 % w/v greatly affected glucose utilization and the rate of ethanol production, until totally shut down glucose utilization and ethanol synthesis in presence of lactic acid 0.6 % (w/v) in glucose-mineral salts medium.

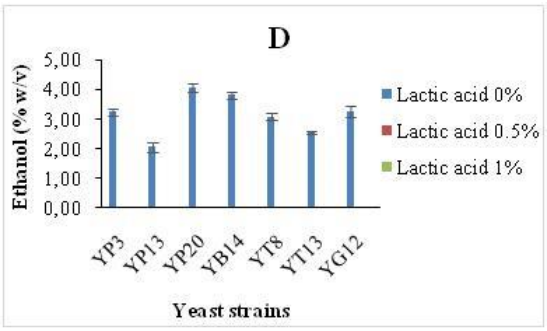

Fig. 4D. Effect of lactic acid concentration on ethanol production of yeasts

However, lactic acid content often attained 0.6 - 0.8 % (w/w) even 1.1 % (w/w) during cocoa fermentation carried out around the world [6, 39]. This indicates that, although yeasts initiate cocoa fermentation process, they can't realize totally fermentative process single in order to obtain cocoa beans good fermented, moreover the lactic acid contribution to obtain fermented dry cocoa beans that give a typical chocolate flavor is more often controversial and considered as not essential to cocoa fermentative process because of its non-volatility. Indeed, the high migration of lactic acid into the beans may contribute to the high acidity of cocoa beans from some geographical regions and poorer chocolate

quality [60]. Thus, cocoa beans with lactic acid exceeding 5 mg/g (even 0.5 % w/w) dry weight tend to have a pH value less than 5.0 and have lesser quality [60, 61, 73].
This issue could be resolved by AAB notably Acetobacter species which oxidize a part of lactic acid produced by the lactic acid bacteria (LAB) into acetic acid and acetoin desirable components for flavor development in fermentation process [69, 74].

3.4. Enzymatic profile and selecting of potential starters

The results of the screening for the presence of specific enzymatic activities of the 7 yeast strains are reported in table 3.

Table 3.
Enzymatic profile and acetoin production of yeast strains

Yeast Strains	β-gluc	Prot	Este	Pecti	Acetoin prod
YP3	-	-	-	-	+
YP13	-	-	-	-	+
YP20	+	-	-	-	+
YB14	+	+	-	+	+
YT8	-	-	-	-	-
YT13	-	-	-	-	+
YG12	+	-	-	-	+

+: positive reaction, – : negative reaction
β-gluc: β-glucosidase, Prot: protease; Este: esterase; pecti: pectinase; acetoin prod: acetoin production

B-glucosidase activity was found by YP20, YB14 and YG12, while protease and pectinase activities were from YB14 only. No yeast strain gave positive result for ester-hydrolase activity.
Protease activity could be beneficial for the microorganisms during the fermentation process, by liberating assimilable nutrient sources, such as amino acids and peptides necessary to microorganisms'growth. Indeed, proteins content in cocoa pulp range of 0.4 to 0.6 % w/w [45]. Thus, the protease activity by YB14 would contribute to rapid microbiota growth in mass fermenting. Moreover, endo and exo protease activities influence the concentration of free amino acids within the beans [16], contributing to chocolate flavor due to the formation of pyrazines during roasting. B-glucosidase has a great potential to be used in various biotechnological processes like liberation of aromas, flavours, and isoflavone aglycones to oligosaccharides and alkylglycosides [75-77]. Indeed, β-glucosidase in yeasts is responsible for the release of the flavoring compounds like nerol, geraniol, linalool, benzyl and phenylethyl alcohols [78]. Thus, β-glucosidase activity produced by YB14 is essential to cocoa fermentation as it will contribute to develop aromas molecules of chocolate. Moreover, this enzyme would contribute to degrade the minimal quantity of cellulosic biomass founded into cocoa pulp.
Pectinases are responsible for the degradation of pectic substances that occur as structural polysaccharides in the middle lamella and the primary cell walls of plant tissues [79]. Some yeasts, as *Kluveromyces marxianus*, produce pectinolytic enzymes which are believed to play a central role in the degradation of the viscous pectin-rich pulp [80, 81]. The action of yeast pectinolytic enzyme is essential for the growth of other microorganisms involving in cocoa fermentation [80]. Indeed, by their depectinization activity, yeasts are responsible for liquefying the cocoa pulp, which causes pulp drainage (release of sweatings) and reduces pulp viscosity that in turn allows air ingress into the fermenting cocoa pulp-bean mass. This aeration of fermenting cocoa pulp-bean mass favorize the growth of AAB and others bacteria necessary to cocoa fermentative process [20]**.** Thus, pectinase activity from YB14 constitutes a potential

from characteristic for this strain in the starter selecting to control cocoa fermentation.

Besides, all 7 strains excepted YT8 are able to produce acetoin (table 3). Thus, the ability of these yeast strains to produce acetoin is a potential way in the aromas precursors' production of chocolate. Indeed, citric acid metabolism lead to produce some molecules aroma such as acetoin (3-hydroxy-2-butanone) which are desirable flavor in fermentation process [82].

Taken together, our results indicate that YB14 and YP13 constitute the potential yeast starters to control cocoa fermentation in Ivory Coast. YB14 showed the best resistance profile through their ethanol production for most parameters used for screening such as temperature, pH, as well as organic compounds stress. Moreover, this cocoa-specific strain showed positive results for almost all the enzymatic activities, particularly β-glucosidase, protease and pectinase. As for YP13, it showed an excellent profile of screening with ethanol and organic acids stress only.

3.5. Phenotypic characterization of YB14 and YP13

Growth was assessed under stress conditions in order to determine the functional properties of selected yeast strains (figures 5 A, B, C, D, E, F, G H, I). The results showed that YB14 and YP13 were able to grow up to 45°C, with the higher tolerance at 40 °C. The data indicate also that YB14 and YP13 showed acid pH tolerance up to pH 2. The both strains showed a good tolerance at pH 3 and pH 4 while at pH 5, YP13 showed a higher growth comparatively to YB14. The ability of these two strains to resist under temperature and stress conditions, as found in cocoa fermentation, constitute the potential properties of these strains to grow and dominate the cocoa fermentative process in Ivory Coast as reported by [83].

Furthermore, growth assessment was also done to investigate the use of different carbon sources, growth in ethanol or organic acids and to test the high osmotic stress tolerance.

The two strains grew well on carbon source as glucose, fructose and sucrose. However, high growth is observed for glucose 2 %, fructose 2 % and sucrose 4 % with the two strains. Concerning growth under ethanol stress, the results indicate that the growth was successively impaired with ethanol raise. Though, YB14 showed alcohol-resistance up to ethanol 12 %, while YP13 showed alcohol-resistance up to ethanol 8 %.

Also, these two yeasts were able to grow in a medium containing citric acid up to 4 %, acetic acid up to 1 % and lactic acid at 1.5 %, respectively.

The figure 5J shows that YB14 and YP3 well grew under high osmotic stress due by glucose 50 % or mixed glucose 5 % with NaCl 10 %, respectively.

The growth of these yeast strains under stress conditions confirm the ability of these yeast strains to be used as starters into many food process notably cocoa fermentation process.

3.6. Molecular characterization

The molecular characterization indicates that YB14 was identified as *Saccharomyces cerevisiae* and YP13 as *Pichia kudriavzevii*.

Saccharomyces cerevisiae already showed some performance as starter in some biotechnological applications of fermentation [8, 84]. Even, *Pichia kudriavzevii* also showed performance asstarter recently in a study leaded by [41].

However, these two-species taken together

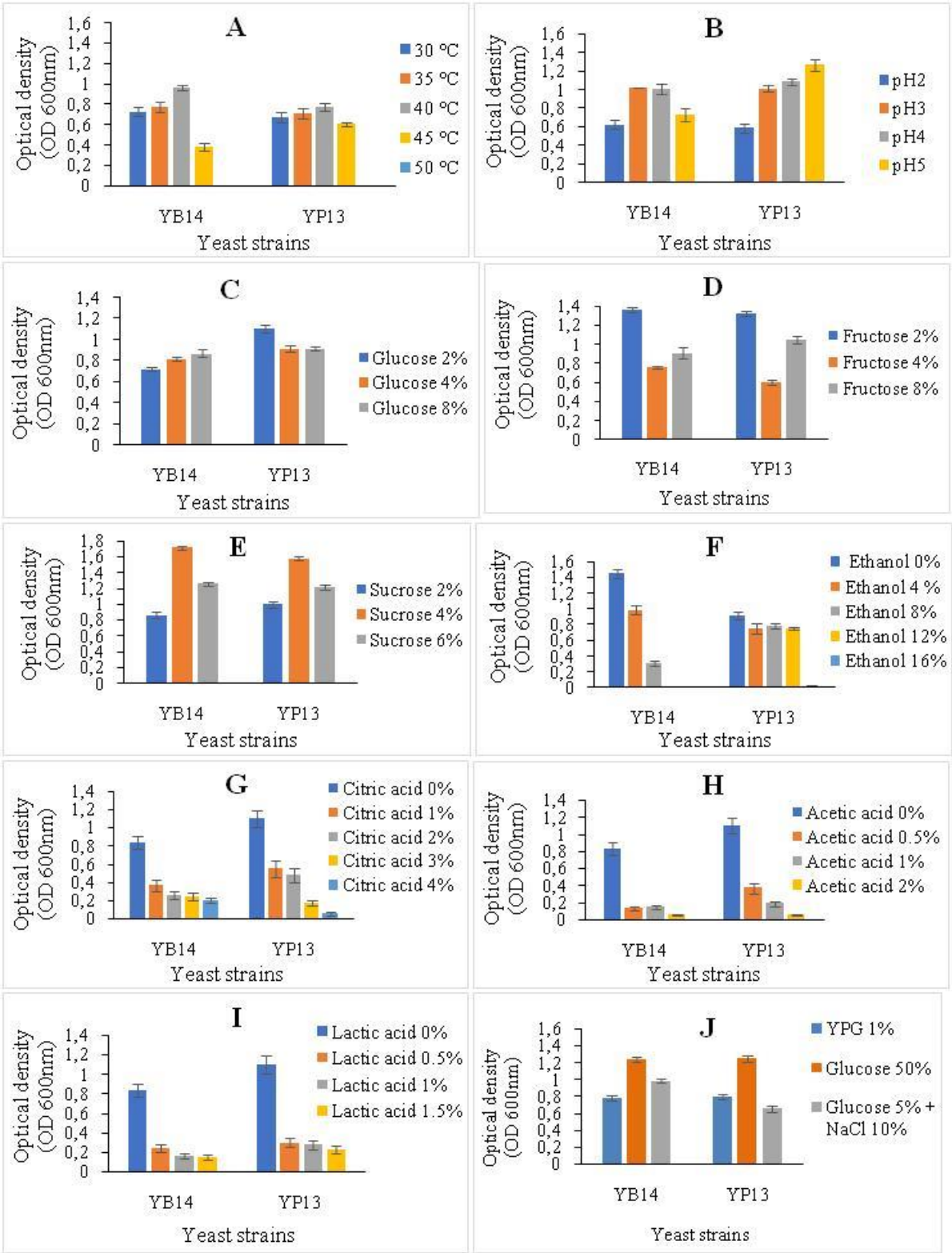

Fig.5. Effects of temperature (A), pH (B), glucose (C), fructose (D), sucrose (E), ethanol (F), citric acid (G), acetic acid (H), lactic acid (I) and high osmotic pressure (J) on yeasts growth.

have never been used in a microbial cocktail. Thus, considering the important potentialities that they release, it should be carried out cocoa fermentation with these two-strains for a control of the Ivorian fermentation process.

4. Conclusion

This study showed that two yeast strains, notably YB14 (*Saccharomyces cerevisiae*) and YP13 (*Pichia kudriavzevii*), with high ethanol producer under stress conditions were able to produce specific enzymes necessary to cocoa fermentation and produce acetoin, which is desirable flavor in fermentation process. Thus, these two strains can be used as starter cultures in cocoa fermentation which may contribute to the control of the cocoa fermentative process in Ivory Coast.

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