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Killer Yeasts Isolated from Ivorian Cocoa Fermentation and their Antagonist Activity on Phytopathogens *Dickeya solani* and *Dickeya dadantii*

SAMAGACI Lamine[1,*], OUATTARA Hadja[1], KOSSONOU Silver[1], KADET Victoria[1], LEMAIRE Marc[2], NIAMKE Sebastien[1]

[1]Laboratory of Biotechnology, Agriculture and Valorization of Biological Resources, Felix Houphouët –Boigny University, P.O. Box 582 Abidjan 22, Ivory Coast Abidjan, Côte d'Ivoire
[2]Laboratoire Microorganism, Adaptation and Pathogenesis, Claude Bernard University, Lyon, France
*Corresponding author: Salamine1983@gmail.com

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Abstract The yeasts involved in cocoa fermentation play an important role in obtaining good cocoa beans' quality. Apart from cocoa fermentation, these yeasts could have a good inhibitory action against the growth of any phytopathogenic microorganism notably *Dickeya dqdantii* and *Dickeya solani.* This study is the first to evaluate the inhibitory action of yeasts involved in cocoa fermentation on the rot potato phytopathogen. For this purpose, the yeasts isolated from Côte d'Ivoire were confronted with the sensitive strain of *Saccharomyces cerevisiae* (W303). Moreover, the yeast killers were used to evaluate their inhibitory action on *Dickeya solani* and *Dickeya dadantii*. Finally, the physicochemical parameters (temperature, pH and salt concentration) capable to influence the inhibitory action of killer yeasts on *Dickeya solani* and *Dickeya* were evaluated. Thirty-three (33) yeasts out of the yeasts isolates tested were capable to produce toxic substances against the sensitive strain of *Saccharomyces cerevisiae* (W303). Among these yeasts, 14 are capable to inhibit the growth of *Dickeya solani* at temperature of 25 °C and 30 °C as well as pH of 4.5 and 6. Four (4) of these yeasts (C1P8, E10P10, D12P12 and E10P19) exert an inhibiting action on the growth of *Dickeya dadantii* in the same condition as before. These four yeasts isolates can be used in the biological fight against these two phytopathogens responsible for potato rot.

Keywords: *Cocoa yeast, Dickeya dadantii, Dickeya solani inhibition activity*

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1. Introduction

Yeasts are microorganisms widely used in biotechnological applications, notably in the manufacture of various products [1] and in the biological fight against pathogen microorganisms of plants and animals. This led researchers to investigate several environments and fermented foods in order to isolate yeasts with interesting functional properties for use a lot of application [2].

Among fermented foods, yeasts involving in cocoa fermentation have been subject of several studies in order to select potential microbial starters for controlling the cocoa fermentation process [3]. Indeed, research on the yeasts isolated in cocoa fermentation has mainly focused either on their microbial diversity [4], their capacity of pectinolytic enzymes production [5] and a few studies on their antagonist activity [6]. The few studies on yeasts Killer in cocoa fermentation carried out in some cocoa production countries notably Côte d'Ivoire, Cameroun and Brazil was intended to isolate yeasts Killer for use as biocontrol agent against undesirables' fungi during cocoa fermentation [6].

However, no study has been interested on the antagonist activity of yeasts killer on the growth of *Dickeya solani* and *Dickeya dadantii* who are fearsome pathogen of potato rot and others commercial plant [7,8,9]. Also, no treatment exists for these phytopathogen microorganism hence the search of killer yeasts capable to be used like antagonist agent for *Dickeya sp.*

The aim of this study is to isolate yeasts killer involving in Ivorian cocoa fermentation, to verify their antagonist activity on the growth of two potato rot pathogen and to determine the optimum condition for their antagonist activity in sight to use this killer yeasts as biocontrol agent.

2. Material and Methods

2.1. Yeasts Isolate Use in this Study

Yeasts previously isolated in cocoa fermentations of eleven cocoa production regions in Côte d'Ivoire were used for this study [4]. Theses isolates were stored in MYGP broth (0.3% (w/v) malt extract; 0.3% (w/v) yeast extract; 0.5% (w/v) peptone; 1% (w/v) glucose) supplemented with 20% glycerol and stored at -80°C for further tests. Moreover, the sensitive strain *Saccharomyces cerevisiae* (W303) and bacteria phytopathogen *Dickeya solani* (DS49) and *Dickeya dadantii* (AS9) isolated from diseased potatoes [7] have been provided by laboratory of microorganism, adaptation and pathogenesis (MAP) of University Claude Bernard of Lyon (France).

2.2. Screening of Yeast Killer Involving in Ivoirian Cocoa Fermentation

2.2.1. Qualitative Assay of Killer Yeast Isolates

The ability of yeasts to produce toxins capable of inhibiting the growth of a sensitive strain was studied using the method described by Woods et al. [10]. A cell suspension of *Saccharomyces cerevisiae* (W303) was firstly prepared in 1 mL sterile distilled water. Then, 40 µL of this suspension was inoculated into Kill-OXO 2X agar [4 mL (4% (w/v) glucose, 2% (w/v) yeast extract, 2% (w/v) peptone, 4% (w/v) agar, 0.003% (w/v) methylene blue) and 4 mL 0.1 M phosphate citrate buffer pH 4.6]. This set was then inoculated with 24 h yeast culture previously cultivated in YPG-agar (1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose) at 30°C. Indeed, the yeast isolate was transferred in sterile fabric using laboratory drum and the fabric with isolate yeast were next transferred in Kill-OXO2X containing the sensitive yeast (w303). After incubation at 30°C during 48 h, the presence of an inhibition halo around the isolates tested indicates the yeasts ability to produce killer toxins.

2.2.2. Quantitative Assay of Killer Yeast Isolates

The method described below to quantify the inhibition activity was used but *Saccharomyces cerevisiae* (W303), *Dickeya Solani* (DS49*)* and *Dickeya dadantii* (AS49*)* as sensitive strain. Also, the wells of 5 mm diameter were made in Kill-OXO2X containing the sensitive and 10 µL of yeast cultures previously made in MYGP broth (incubated at 30°C during 24 h) with optical density 1 was used to inoculated the wells [11]. The culture was incubated at 30°C for three days. Inhibitory activity was calculated using the following formula: D = (5logA) x 10, where D is the diameter of the inhibition zone (in mm), and A is the inhibitory activity in $AU.mL^{-1}$ [12,13].

2.3. Enzyme Production of Killer Yeasts Involving in Côte d'Ivoire Cocoa Fermentation

The production of amylase, cellulase and polygalacturonase activities of yeasts killer was carried out using the method of Tansel et al. [14] with some modifications. A minimum medium (peptone 0.5%; yeast extract 0.5%; MgSO4.7H2O 0.05%; FeSO4 .7H2O 0.001%; NaCl 0.001%; agar 1.5%) was used for the culture. To this medium, 1% carbon substrate (starch for amylase, carboxymethylcellulose (CMC) for cellulase and polygalacturonic acid (PGA) for polygalacturonase) was added. Yeast killer was spot-seeded onto the agar. After incubation at 30°C for three days, a solution of lugol (solution iodo-iodurée: 0.1% I2 + 1% KI) was added to the medium to reveal enzymatic activity. As lugol complexes with macromolecules to give a specific color, a bright zone (halo) appears around colonies that have degraded the carbon source.

Proteolytic activity was demonstrated using milk agar made from 5% skimmed milk and 1% agar [15]. Killer yeast colonies were spotted and the culture incubated at 30°C for three days. Degradation of milk casein is characterized by direct visual observation of a clear, transparent zone around the colony [12,13].

2.4. Influence of Temperature, Ph and Nacl on the Antagonist Activity of Killer Yeast on the Growth of *Dickeya Solani* and *Dickeya Dadantii*

The method described in 2.2 was use to evaluate the influence of temperature, pH and NaCl on the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49)., For this study, the pH of Kill-OXO 2X was adjusted 4.5; 6; 7, for temperature the culture were incubated at 25, 30, 35°C and for salinity study the NaCl 2 % was added in Kill-OXO 2X.

3. Results

3.1. Screening of Yeasts Killer Isolated from Cocoa Fermentation in Ivory Coast

On the 11 cocoa production regions, in Côte d'Ivoire, the yeasts isolated from 5 regions didn't have inhibition action on the growth of sensitive yeast *Saccharomyces cerevisiae* (w 303). On the other hand, 33 yeasts strains isolated of 6 others regions gave inhibition action on the growth of sensitive yeast (W303) with respectively 12 yeasts strains isolated from Nawa region, 9 strains for Sud Comoe, 6 strains for Gboble, 3 strains for Tonkpi, 2 strains for Guemon (2) and 1 strain for Goh Table 1.

Table 1. Killer yeasts isolated from Ivorian cocoa fermentation in different cocoa cultivation areas

Area	Yeasts isolated	Killer yeasts
Nawa	129	12
Sud Comoe	218	9
Gboble	130	6
Tonkpi	129	3
Guemon	136	2
Goh	130	1
Indenie	129	0
Sans Pedro	133	0
Lohdjiboua	271	0
Haut Sassandra	130	0
Cavally	137	0

3.2. Killer Yeasts Isolated from Ivorian Cocoa Fermentation Capable to Kill *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49)

On 33 killer yeasts strains isolated from Ivoirian cocoa fermentation, 14 Killer yeasts have inhibition action on the growth of phytopathogen bacteria *Dickeya solani* (DS49). Among, these killer yeasts which are capable to kill *Dickeya solani*, 4 strains have also inhibition action on the growth of phytopathogen bacteria *Dickeya dadantii* (AS49) (Table 2).

Table 2. Inhibition activity of killer yeasts isolated from cocoa fermentation on *Dickeya solani* and *Dickeya dadantii*

Killer yeasts	*Dickeya solani* (DS49)	*Dickeya dadantii (A49)*
C1P8	+	+
E10P10	+	+
E10P19	+	+
D12P12	+	+
D11P10	+	-
H9P8	+	-
G1P9	+	-
F7P10	+	-
D5P12	+	-
E2P19	+	-
E3P12	+	-
F8P10	+	-
E7P8	+	-
E3P19	+	-
C8P10	-	-
A12P12	-	-
B4P12	-	-
B10P12	-	-
C5P12	-	-
H3P8	-	-
H4P8	-	-
F2P10	-	-
F5P10	-	-
D7P12	-	-
E5P12	-	-
E4P19	-	-
E4P12	-	-
E12P10	-	-
A6P12	-	-
D8P12	-	-
F1P10	-	-
H7P8	-	-
H11P8	-	-

3.3. Enzyme Production of Killer Yeasts Isolated from Ivoirian Cocoa Fermentation Performing Inhibition Action on *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49) Growth

The Killer yeasts involving in cocoa fermentation in Côte d'Ivoire and capable to kill bacteria phytopathogen *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49) produce a diversity of enzymes except C1P8, E10P19 and F8P10 which didn't produce any enzyme. However, the other killer yeasts have produced at least 2 types of enzymes. Moreover, among the killer yeasts capable to inhibit the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49), two (2) isolates (E10P10 and D12P12) produce amylase and protease while the other two isolates (E10P19 and C1P8) didn't produce none enzyme tested (Table 3).

Table 3. Hydrolytic enzymes production of killer yeast capable to inhibit *Dickeya solani* or *Dickeya dadantii*

Killer yeasts	Enzym			
	Amylase	Cellulase	Polygalacturonase	Protease
H9P8	+	+	-	-
D5P12	-	+	+	-
D11P10	-	+	-	-
E7P8	+	-	+	-
E10P10	+	-	-	+
E2P19	+	-	-	-
F7P10	+	-	-	-
E3P12	+	-	-	-
E3P19	+	-	-	+
D12P12	+	-	-	+
E10P19	-	-	-	-
F8P10	-	-	-	-
G1P9	-	-	-	+
C1P8	-	-	-	-

3.4. Influence of Temperature, pH and Nacl on Inhibition Activity of Killer Yeasts Isolated from Ivoirian Cocoa Fermentation

3.4.1. Effect of Temperature on Antagonist Activity of Killer Yeast

The results show the relative inhibitory activity of the killer strains at 35°C is negligible. These killer yeasts cannot exert any inhibition action on the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49) at 35 °C. While, at 25 and 30° C the most of killer yeasts are capable to inhibit the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49) except C1P8, E10P19, G1P9, E2P19, F8P10 and E7P8 which have not inhibition action on *Dickeya solani* (DS49) at 25 °C (Figure 1 and Figure 2)

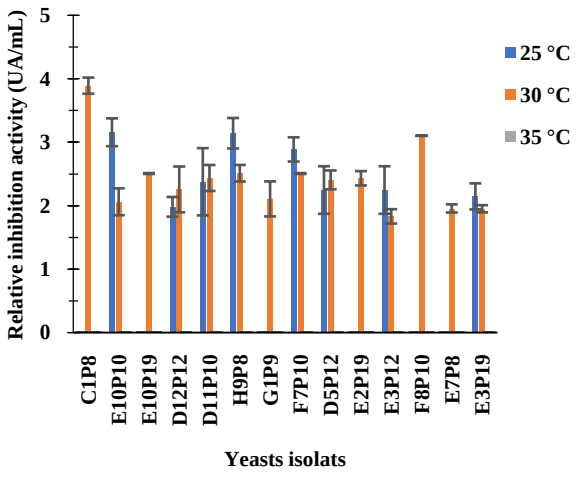

Figure 1. Inhibition activity of Killer yeast on *Dickeya solani* at different temperatures

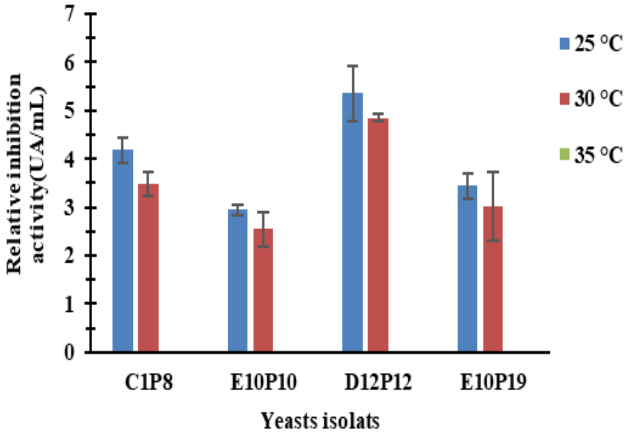

Figure 2. Inhibition activity of Killer yeast on *Dickeya dadantii* at different temperatures

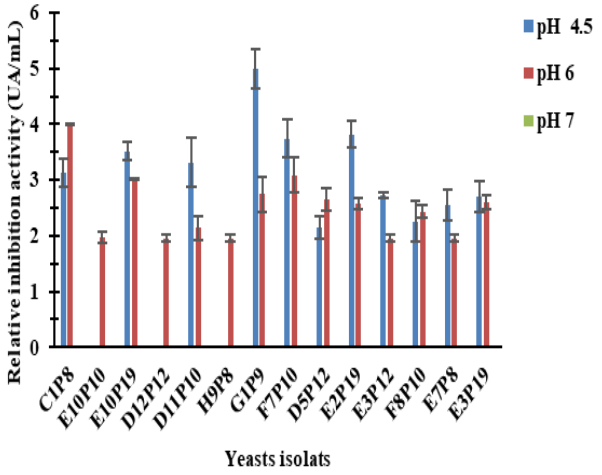

Figure 3. Inhibition activity of the Killer yeast isolated from cocoa fermentation on *Dickeya solani* at different pH

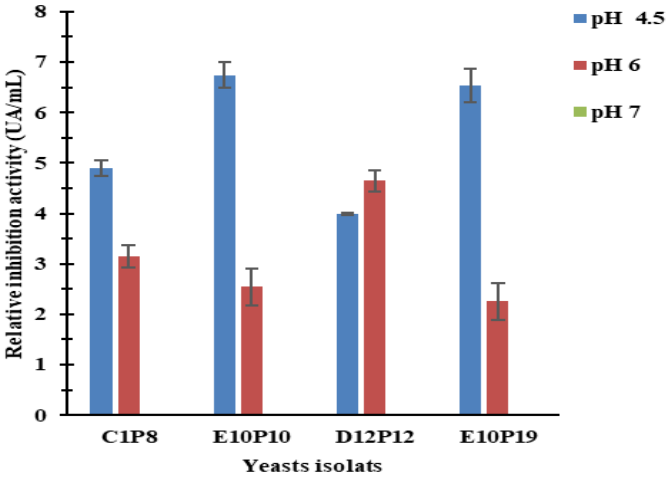

Figure 4. Inhibition activity of the Killer yeast isolated from cocoa fermentation on *Dickeya dadantii* at different pH

3.4.2. Effect of pH on Inhibition Activity of Killer Yeasts Isolated from Ivorian Cocoa Fermentation

As at 35°C, the killer yeasts are not inhibition action on the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (A49) at pH 7. However, the killer yeasts kill *Dickeya solani* (DS49) and *Dickeya dadantii* (A49) at pH 5 and 6 except E10P10 and E7P8 which can't kill these strains at pH 5 (Figure 3 and Figure 4). Moreover, the killer yeasts E10P10 and E10P19 with relative inhibition activity more than 6 UA/mL are more inhibition activity on the growth of *Dickeya dadantii (A49)* at pH 4.5 (Figure 4 While, F7P10 exert more antagonist action (5.25 UA/mL) on the growth of *Dickeya solani* (DS49) at pH 4.5 (Figure 3).

3.4.3. Effect of Nacl on Inhibition Activity of Killer Yeasts Isolated from Ivoirian Cocoa Fermentation

The killer yeasts lose their inhibition actions on the growth of *Dickeya solani* (DS49) and *Dickeya dadantii* (AS49) in presence of NaCl at 2% except E10P10, D12P12 which preserve their antagonist action on *Dickeya dadantii* (A49) (Table 4 and Table 5).

Table 4. Inhibition activity of Killer yeast isolated from cocoa fermentation on *Dickeya solani* on salty medium

Killer yeasts	Relative inhibition activity (UA/ml)	
	Kill-oxo	Kill-oxo + 2%NaCL
C1P8	3.39±0.59	0
E7P8	2.06±0.15	0
H9P8	2.76±0.26	0
G1P9	1.95±0.04	0
D11P10	2.43±0.14	0
E10P10	1.95±0.1	0
F7P10	2.51±0.2	0
F8P10	2.9±0.4	0
D5P12	2.4±0.1	0
D12P12	2.43±0.08	0
E3P12	1.83±0.2	0
E2P19	2.2±0.3	0
E3P19	1.95±0.04	0
E10P19	2.25±0.26	0

Table 5. Inhibition activity of Killer yeast isolated from cocoa fermentation on *Dickeya dadantii* on salty medium

Killer yeasts	Relative inhibition activity (UA/ml)	
	Kill-oxo	Kill-oxo + 2% NaCL
C1P8	3.13±0.18	0
E10P10	2.64±0.3	3.11±0.2
D12P12	4.36±0.43	4.06±0.31
E10P19	2.23±0.28	0

4. Discussion

Killer yeasts are increasingly sought after as a biocontrol agent against undesirable fungi in biotechnological applications. The yeasts which produce a toxic substance have been isolated from several fresh fruits (dates, grapes, figs and strawberries), juices (carrot, orange, sugar cane and tomato) and fermented food (meat, fish, cocoa). Concerning cocoa fermentation, some studies have been able to isolate killer yeasts [6] as was the case in the present study. We have had 33 isolates capable to produce a toxic substance out of 1672 isolates tested either a rate of 1.97 %. This rate is low unlike that found by de Lima et al. [16] which is 5 % of killer yeasts isolated from tropical fruits. However, these killer yeasts could be very interesting in the cocoa fermentation process. During this process, fungal germs such as ochratoxin-producing moulds develop, which are undesirable germs and could constitute a public health problem. The use of these killer yeasts in cocoa fermentations would be an advantage, as they could inhibit the growth of these ochratoxin-producing moulds.

Moreover, the yeasts isolated from cocoa fermentation of Côte d'Ivoire produce different types of enzymes (amylase, polygalacturonase, cellulase and protease) which can have technological role in several manufacturing processes. Indeed, D5P12 and E7P8 which produce polygalacturonase and amylase or cellulase can play a very important role during cocoa fermentation. The polygalacturonase activity is involved in the degradation of pectin contained in cocoa pulp. The degradation of cocoa pulp is necessary and important to obtain a good quality of cocoa beans [17]. In addition, these two (2) yeasts killer with H9P8 having amylase and or cellulolytic activity can be exploited in the fermentation of Ivoirian cassava food attieke. Indeed, the cellulase produced by these yeasts could play a role in softening of cassava and the amylase could hydrolyze the starch contained in cassava into free sugar. These sugars can be used as a substrate by other microorganisms involved in the fermentation process of Ivorian fermented food (attieke) in particular bacteria of genus bacillus and lactic acid [18, 19, 20].

Also, the antagonist activity of killer yeasts can be expressed by production of toxic substance or the production of exoenzyme in particular protease. The protease activity of yeasts killer E10P10; E3P19; D12P12 and G1P9 can be linked to their antagonist acitivity. Indeed, According to Pretscher et al. [21] the capacity of the *Aureobasidium pullulans* to produce alkaline protease allow the reduction of spore germination and length of *Penicillium expansum* and *B. cinera*. Moreover, according some studies, the inhibition action of protease will be linked to the disruption of the structure of the membrane of sensitive microorganisms by digesting the proteins embedded in this membrane [1,22].

In addition, the antagonist activity of yeasts killer on the growth of *Dickeya solani* and *Dickeya dadantii* depends of environnementale conditions (temperature, pH and NaCl). This could be linked to the fact that according Sainz et al. [23] environmental condition are factors that influence the behavior of yeasts, in particular their ability to grow and produce metabolites. Also, several studies have demonstrated that salt at 2% delete the inhibition action of some microorganisms [24] firstly and on the other hand, the major strains produce the toxic substance at 25 and 30°C at pH 4.5 as in the case of our study [1].

Among yeasts killer, four (4) (C1P8, E10P10, D12P12 and E10P19) have a good inhibition activity on the growth of *Dickeya solani* and *Dickeya dadantii* at temperature included 25 and 30°C and pH included 4.5 at 6. Indeed, those yeasts isolates could be used in biological control of these two (2) redoubtable phytopathogen strains of *Dickeya sp* which are responsible for causing disease in numerous agricultural crops [8] and ornamental plants as *candida oleophila* first yeast marketed as biocontrol agent [25].

5. Conclusion

Few yeasts involving in the cocoa fermentation of Côte d'Ivoire are capable to produce toxic substances against the growth of the strain of *Dickeya solani* and *Dickeya dadantii*, two bacteria involved in potato soft rot. Also, these yeasts killer have produced a diversity of exoenzyme and their inhibition action depend of environmental conditions (temperature, pH and NaCl). Four (4) (C1P8, E10P10, D12P12 and E10P19) yeasts killer could be used as biocontrol agent against *Dickeya*

solani and *Dickeya dadantii*.

Conflict of Interest

Authors declare that they do not have any conflict of interest.

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