*Original Research Article*

Effect of inoculum size in fermentation of cocoa beans using *Candida tropicalis*

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ABSTRACT

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| Cocoa fermentation is a crucial step in determining the quality, yet spontaneous fermentation often yields inconsistent results. This study aimed to evaluate the effectiveness of *Candida tropicalis* as a starter culture to improve fermentation performance and bean quality at a semi-pilot scale (10 kg). Fermentation trials were conducted using five treatments (control and *C. tropicalis* at 10³, 10⁴, 10⁵, and 10⁶ cells/g) on 10 kg of fresh cocoa beans, with samples collection every 24 hours for six (6) days. Key parameters measured included fermentation index (FI), pH and acidity of pulp and cotyledon, and reducing and total sugars in both bean pulp and cotyledon. The results showed that inoculation with *C. tropicalis*, particularly at 10⁵ and 10⁶ cells/g, significantly improved the speed and quality of fermentation. The highest FI values (≥1.65) and brown bean percentages (≥87%) were achieved after just 96 hours in these treatments, compared to 144 hours in the control. The pH, acidity, sugars content also showed considerable improvement with best strains. These findings suggest that *C. tropicalis*, especially at 10⁵ cells/g, can enhance fermentation efficiency, reduce processing time by up to 50%, and improve bean quality. Future research should explore the sensory impact of this yeast on chocolate flavor, optimize fermentation protocols, and assess scalability for industrial applications. |

*Keywords: (Candida tropicalis, cocoa fermentation, yeast starter, cocoa quality)*

1. INTRODUCTION

Cocoa fermentation is an essential process for obtaining high-quality cocoa beans, which are required for chocolate manufacturing, cocoa powder, cocoa butter, and other cocoa-based beverages that we consume or use in our daily lives (Guehi et al., 2010; Guzmán-Alvarez and Márquez-Ramos, 2021). It occurs naturally and spontaneously with the help of various microorganisms, primarily yeasts, lactic acid bacteria, and acetic acid bacteria (Afoakwa et al., 2013; Apriyanto and Umanailo, 2019). However, due to its spontaneous nature, fermentation is often inconsistent, leading to significant variability in bean quality and causing economic losses.

To improve consistency in bean quality and ensure successful fermentation, starter cultures are used to control the fermentation process. Yeasts play a crucial role among the microorganisms used as starters~~,~~ in cocoa fermentation experiments. Some yeast species have been extensively studied, including *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and *Hanseniaspora* spp., due to their ability to enhance the fermentation process and influence flavour development (De Vuyst and Leroy, 2020; Díaz‐Muñoz and De Vuyst, 2022; Gutiérrez-Ríos et al., 2022). These yeasts contribute to the degradation of pulp sugars, promote alcohol production, and facilitate the growth of acetic acid bacteria, which are essential for proper fermentation and flavor formation (Alvarez, 2017; Assi-Clair et al., 2019).

Apart from these commonly studied yeasts, non-*Saccharomyces* species also play a significant role in cocoa fermentation. One such species is *Candida tropicalis*, which has been identified as an important actor in the fermentation process (Ardhana M. M. and Fleet G. H, 2003; Kadet et al., 2024; Mahazar et al., 2015). Unlike *Saccharomyces cerevisiae*, *C. tropicalis* exhibits unique metabolic properties that may influence the development of specific aroma compounds and improve fermentation efficiency. Despite its potential, research on *C. tropicalis* as a starter culture remains limited. The only known fermentation trial involving *C. tropicalis* was conducted by (Jamili et al., 2014), who used the yeast for a fermentation mass of 350 g. The results of this study showed that a higher percentage of brown beans was observed in the yeast starter fermentation trial as compared to the control. However, no studies have explored its impact on larger fermentation masses to evaluate its effectiveness in improving bean quality on a semi-pilot or industrial scale.

The objective of this study is to determine the impact of *C. tropicalis* as a starter culture on the fermentation process and the quality of dried fermented cocoa beans at a semi-pilot scale.

2. material and methods

**2.1 Materials**

The *Candida tropicalis* strain used in this study was isolated from fermenting cocoa in Côte d’Ivoire from a previous study conducted at the Research Unit of Felix Houphouët Boigny University. The plant material in this study consisted of cocoa pods collected from farmers in the Agboville region, which is located at 79 km of Abidjan, in southern Côte d’Ivoire. Fermentation took place at the National Center for Floristics Félix Houphouët-Boigny University in Abidjan.

**2.2 Methods**

**2.2.1 Determination of the microbial load of the starter culture for the fermentation trials**

The frozen starter powder was first revived, checked for viability and purity before being used for fermentation. To do that, an amount of 0.1 g of the starter powder removed from the freezer was resuspended in 0.9 mL of peptone water. Then, a volume of 25 μL of each suspension in addition to 25 μL of methylene blue was deposited on the Thoma cell. Microorganisms were enumerated using microscope (G×40). The number of live cells per millilitre was then determined by the following formula:

 **N= n x 5 x 105 x fd**

n : Average number of cells counted per chosen square

N : Number of cells per milliliter

fd : Dilution factor

Various calculations were performed to achieve cell concentrations of 10³, 10⁴, 10⁵, and 10⁶ cells/g to inoculate 1 kg of fermenting cocoa bean.

**2.2.2 Fermentation trials of cocoa beans**

Fermentation trials were conducted in farm conditions using banana leaves. The pod was opened manually with the help of a machete. They were then weighed to obtain 10 kg of cocoa bean mass. The amount of starter powder determined in 2.2.1 was added to each sample respectively. In total 5 trials were carried out in …..including the control. The table 1 below summarizes the trials. Fermentation was conducted for 6 days with mixing and sample collection every 24hs. After collecting samples various analysis was performed.

**Table 1. Summary of fermentation trials conducted**

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| --- | --- |
| **No** | **Samples**  |
| **1** | Spontaneous fermentation (control)  |
| **2** | *Candida tropicalis* 10³cells/g |
| **3** | *Candida tropicalis* 10⁴ cells/g  |
| **4** | *Candida tropicalis* 10⁵cells/g |
| **5** | *Candida tropicalis* 10⁶cells/g  |

**2.2.2.1 Evaluation of Fermentation Index of bean cotyledon**

The fermentation index was determined using the method of Gourieva and Tserrevitinov (1979). An amount of 0.5 g of cocoa cotyledons, previously ground with a blender, was accurately weighed. A 97:3 (V/V) mixture of methanol (97%) and hydrochloric acid (3%) was prepared, and 50 mL of the solution was added to the ground cotyledons. The mixture was refrigerated at 8°C for 17 hours before being filtered through Whatman filter paper. The fermentation index (FI) was calculated as the ratio of absorbance at 460 nm to that at 530 nm. Three independent measurements were performed, and the average results were recorded. A good fermentation index is obtained between 1 and 1.4.

**2.2.2.2 Determination of pH and acidity of the bean pulp**

The acidity of the bean pulp was measured by weighing 20 g of cocoa beans pulp in 20 mL of distilled water. The resulting mixture was thoroughly stirred and then filtered using Whatman filter paper. After filtration, the pH of the solution was measured by immersing the electrode of a pH meter, and the pH value was read directly from the display. Subsequently, a volume of 5 mL of the filtrate was measured and titrated against a NaOH solution (0.1 N) after the addition of two (2) drops of phenolphthalein, until a persistent pink colour was obtained. The following formula is used to determine the acidity of the pulp of fermented cocoa beans (AOAC., 1990; Crafack et al., 2013).

$$A (meq/g)=\frac{V(NaOH)×N(NaOH)×100}{m×V(filtrate)}$$

**2.2.2.3 Determination of pH and acidity of bean cotyledon**

Cocoa bean was hand-pulped, and the resulting cotyledon was ground using an electric blender. Then, two (2) grams of the crushed material weighed using a precision scale were added to 18 mL of distilled water. The mixture is homogenized and then filtered using a Whatman paper with a porosity of 0.45 μm. The pH of the solution was then read using a pH meter. Next, a volume of 5 mL of the filtrate was titrated with a NaOH solution (0.1 N) after the addition of two (2) drops of phenolphthalein, until a persistent pink colour was obtained. The formula used to determine the pulp acidity was the same for determining bean cotyledon acidity.

**2.2.2.4 Evaluation of reducing sugar and total sugar content in bean pulp**

**• Extraction of water-soluble sugars from the pulp**

The extraction of water-soluble sugars contained in cocoa pulp was carried out according to the method of Agbo et al. (1985). A few cocoa beans were randomly selected and manually depulped. Then, five (5) grams of pulp were weighed and introduced into a 200 mL volumetric flask containing 50 mL of distilled water heated to 60 °C. The mixture was stirred until completely cooled and filtered using Whatman filter paper. The filtrate was collected in a 100 mL volumetric flask and diluted to the mark with distilled water.

**• Determination of reducing sugars in the bean pulp**

The quantification of reducing sugars in cocoa pulp was performed using the Bernfeld method (1955). A volume of 100 μL of the previously prepared water-soluble sugar extract was added to test tubes along with 200 μL of DNS reagent. The mixture was vortexed, then heated in a boiling water bath at 100 °C for 5 minutes. After cooling, 2 mL of distilled water was added to the mixture, and the optical density was measured at a wavelength of 540 nm using a spectrophotometer. A blank sample, prepared without sugar extract, was treated under the same conditions. A calibration curve was established using a glucose stock solution at a concentration of 1 mg/mL. The amount of reducing sugars in the cocoa pulp was determined using the regression equation derived from the standard curve.

**• Determination of total sugars in the bean pulp**

The total sugar content in the cocoa pulp was determined using the phenol-sulfuric acid method described by Dubois et al. (1956). A volume of 100 μL of the previously prepared water-soluble extract was placed in test tubes. Then, 1 mL of concentrated sulfuric acid (95–97%) and 200 μL of 5% phenol (w/v) were successively added. After standing in the dark for 15 minutes to cool, 2.7 mL of distilled water was added to the mixture. The optical density was measured at 490 nm against a blank prepared under the same conditions. A standard curve was prepared using a glucose stock solution at a concentration of 1 mg/mL. The total sugar content in each sample was determined using the regression equation obtained from the standard curve.

**2.2.2.5 Evaluation of reducing sugar and total sugar content in bean cotyledon**

**• Extraction of water-soluble sugars from cocoa bean cotyledons**

The extraction of water-soluble sugars from fermenting cocoa cotyledons was performed according to the method of Agbo et al. (1985). Five (5) grams of cotyledons were ground using a blender (Moulinex). Then, 50 mL of distilled water heated to 60 °C was added to the paste and allowed to cool. After cooling, the extract was filtered using Whatman filter paper. The filtrate was made up to 100 mL with distilled water.

**• Determination of reducing and total sugars in cocoa bean cotyledons**

The determination of reducing and total sugars in the cotyledons of fermenting cocoa beans was carried out following the same procedure as described for the bean pulp.

**2.2.2.6 Evaluation of cut test of fermented beans after drying**

The cut test is used to assess the sanitary and fermentation quality of cocoa beans by determining the percentage of defective beans and their level of fermentation. It was conducted following the method described by (Hii et al., 2011). A total of 100 dried fermented beans were randomly selected from each sample. Each bean was sliced lengthwise to maximize surface exposure and examined under daylight to evaluate its color. The beans were then classified, and the percentage of brown beans was calculated and expressed as percentage. The cut test was conducted each day during 144 days of fermentation.

**2.2.3 - Statistical data analysis**

All the experiments were conducted thrice. Analysis of variance (ANOVA) was used to identify significant differences between treatments. The dataset collected during the experiment was thoroughly examined and analyzed using RStudio 2024.

3. results and discussion

3.1 Results

3.1.1 Fermentation index of the bean cotyledon

Figure 1 presents the fermentation index of bean cotyledons over 144 hours. The fermentation index steadily increases over time for all inoculation levels and the control. The fermentation index ranged from (0.379±0.003) and (1.655±0.001). There is also an increase over time across all groups as the fermentation time increases. Higher inoculum levels (*C. tropicalis* 10⁵ and 10⁶) show the most rapid and significant increase in fermentation index, reaching the highest values by the end of the fermentation period (1.655±0.0.01 and 1.296±0.001). Lower inoculum levels (*C. tropicalis* 10³ and 10⁴) exhibit a slower increase compared to the higher levels. All inoculum charges including the control have fermentation index that within 1 and 1.4 after 72-120 hours.

Fig. 1. Evolution of the fermentation index in the bean cotyledon

3.1.2 Evolution of pH and acidity of the bean pulp

The figure 2 below shows the variation of pH and acidity in cocoa bean pulp during 144 hours of fermentation using different concentrations of *Candida tropicalis* (10³ to 10⁶ cells/g) compared to a control (spontaneous fermentation). In general, across all inoculum concentrations and the control, there is a gradual increase in pH of the bean pulp in (A) over time and a gradual decrease in bean pulp acidity over time (B) during fermentation. Higher yeast concentrations, especially 10⁵ and 10⁶, led to a faster and more significant pH rise from 3.2±0.01 at the start to 8.5±0.01 at 144 h and a sharper reduction in acidity, from 0.55±0.003 meq/g to around 0.05±0.005 meq/g. *C. tropicalis* 10⁴ and 10⁶ were particularly effective in accelerating acid breakdown and pH increase. An inverse correlation was observed: as acidity decreased, pH increased, indicating that higher yeast inoculation enhances fermentation efficiency through more active acid metabolism.

Fig. 2 . Evolution of pH and acidity of the bean pulp of cocoa

1. *pH of bean pulp (B) acidity of bean pulp*

3.1.3. Evolution of pH and acidity of bean cotyledon of cocoa beans

Fig. 3 shows the evolution of pH of bean cotyledon (A) and the acidity of bean cotyledon (B) during fermentation trials over a period of 144 hours. Overall, there is a decrease across all pH as time increases in all treatments over the 144 hours of fermentation in graph (A). Initially, all samples start at a similar pH of around 6.73±0.01, indicating a neutral to slightly acidic internal environment. Over time, the pH steadily decreases in all treatments, reaching final values between 4.16±0.01 and 4.63±0.01 at 144 hours. Notably, the lowest pH (4.16±0.01) is observed with *C. tropicalis 10⁵*, indicating greater acid penetration into the cotyledon, while the control and other treatments stabilize at slightly higher levels between (4.45±0.01 to 4.63±0.01). Graph (B) presents the evolution of acidity in the cocoa bean cotyledon during 144 hours of fermentation, using different concentrations of *Candida tropicalis* (10³ to 10⁶ CFU/g) against a control without any inoculum. In contrast to graph (A), which showed a gradual decrease in cotyledon, graph (B) reveals a progressive increase in acidity over time for all treatments. The most significant increase is observed with *C. tropicalis* 10⁵, which reaches the highest acidity (0.16±0.01 meq/g) at 144 hours, confirming the strong acid diffusion previously indicated by its lower pH. These combined results illustrate that higher acid levels inside the bean correlate with lower internal pH, and that *C. tropicalis* 10⁵ promotes the most intense acidification of the cotyledon.

Fig. 3 . Evolution of pH and acidity of bean cotyledon

1. *pH of bean cotyledon (B) acidity of bean cotyledon*

3.1.4 Evaluation of reducing sugar and total sugar content in bean pulp

The reducing sugar content in bean pulp and the total sugar content of bean pulp are represented by figure 4 in (A) and (B) respectively. The reducing sugar content of bean pulp represented by figure (A) shows a rapid decrease in reducing sugar from 0.10±0.003 g/g to 0.00±0.00 hours across all conditions. After 48 h, sugar levels fall to 0.02 g/g in most treatments. Between 72 h and 120 h, values stabilize below 0.01 g/g. At 144 h, all samples show minimal sugar close to 0.00 g/g, indicating complete consumption of sugars in the pulp. The total sugar content of bean pulp represented by figure (4B) shows a decrease in total sugar from 0.50±0.012 g/g to 0.006±0.00 g/g for all samples. By 48 h, sugar levels drop between 0.06±0.01 to 0.13±0.03 g/g across most conditions. At 144 h, total sugar ranges from 0.006±0.00 g/g to 0.05±0.01 g/g in all samples. Overall, total sugar levels start higher and decline more gradually as compared to reducing sugars which were almost completely consumed by 144 h, while total sugars remained between 0.006±0.00 g/g to 0.05±0.01 g/g.

Fig 4. Evolution of reducing sugar and total sugar content in bean pulp

1. *reducing sugar content (B) total sugar content*

3.1.5 Evaluation of reducing sugar and total sugar content in bean cotyledon

The evolution of reducing sugar and total sugar content in bean cotyledon are represented by figure 5A and 5B respectively. Figure 5A shows a steady increase in reducing sugar in bean cotyledons from 0.005±0.001 g/g at 0 h to 0.11±0.01 g/g at 144 hours for *C. tropicalis* 10⁵. At 120 h and 144 h, *C. tropicalis* 10⁵ shows a marked rise, suggesting higher sugar diffusion into cotyledons. The control and other treatments show more gradual increases, obtaining a final value around 0.05 g/g. Figure 5B shows fluctuations in total sugar content in bean cotyledons, starting at 0.23±0.008 g/g across all conditions at 0 h. At 24 h, *C. tropicalis* 10⁵ increases sharply to 0.29±0.02 g/g, while other treatments decrease. From 96 h to 144 h, *C. tropicalis* 10⁵ maintains higher levels of around 0.29±0.02 g/g, unlike other treatments which decline. Overall, *C. tropicalis* 10⁵ promotes the highest and most sustained increase in total sugar in cotyledons. Compared to the reducing sugars, total sugars in cotyledons are consistently higher while reducing sugars steadily increase, total sugars fluctuate more, especially with *C. tropicalis* 10⁵.

Fig 5. Evolution of reducing sugar and total sugar content in bean cotyledon

1. *reducing sugar content (B) total sugar content*

3.1.6 Evaluation of physical quality of cocoa beans

Figure 6 below illustrates the percentage of brown fermented, dried cocoa beans every 24 hours for a period of 144 hours. In general, as fermentation time increases, the percentage of brown beans also increased across all samples. The highest percentages of brown beans were recorded at 144 hours for all the samples, ranging between 62±10% and 87±1%. Notably, fermentation with the inocula *C. tropicalis* 10⁵ and 10⁶ achieved 60% brown beans after just 96 hours, while the control required the full 144 hours to reach a similar result. Inoculum with lower concentrations (*C. tropicalis* 10³ and 10⁴) achieved 60% brown beans after 120 hours of fermentation.

Fig. 6. Percentage of brown beans in fermented dried cocoa seeds

3.2 Discussion

This study investigated the effect of *Candida tropicalis* inoculation on cocoa bean quality during fermentation, with a focus on improving the consistency and efficiency of the process. Cocoa bean quality is commonly assessed by the degree of fermentation, primarily through the cut test and the Fermentation Index (FI), which provide qualitative and quantitative insights respectively (Afoakwa et al., 2013; Dopgima et al., 2023). The cut test, based on visual assessment of bean color, distinguishes between unfermented (slaty, grey), under-fermented (partially purple), and well-fermented (brown) beans (Kongor et al., 2016). However, this method can be subjective due to its reliance on visual perception, necessitating a more objective metric such as the FI, which quantitatively measures anthocyanin degradation during fermentation (Ziegleder, 2017).

In this experiment, the control group underwent natural, spontaneous fermentation, while other groups were inoculated with varying concentrations of *C. tropicalis*. According to (Ngangue et al., 2022), FI values ranging from 1 and 1.40 denote satisfactory fermentation. In this study, all inoculated groups reached or exceeded this threshold, indicating improved fermentation. This was corroborated by the increased proportion of brown beans observed in the cut test. Notably, higher inoculum concentrations (10⁵ and 10⁶ cells/g) resulted in significantly enhanced fermentation outcomes, both in terms of FI values and cut test results. When benchmarked against the Malaysian Standard, which defines well-fermented beans as those with at least 60% brown coloration (Dopgima et al., 2023), the effectiveness of *C. tropicalis* inoculation became even more evident. While spontaneous fermentation reached just 62 ± 10% brown beans by day 6, inoculated samples surpassed this benchmark more rapidly in 96 days (4 days), demonstrating that controlled microbial inoculation can substantially accelerate the fermentation process.

Fermentation progression was also faster with higher inoculum levels. At 10⁶ cells/g, full fermentation was achieved in 72 hours (3 days) according to the FI, and 96 hours (4 days) based on the cut test. Similarly, for 10⁵ cells/g, the FI reached the threshold at 72 hours, with full fermentation visually confirmed by 96 hours. In contrast, lower concentrations (10³ and 10⁴ cells/g) and the control required longer fermentation durations—up to 120–144 hours. The best fermentation performance, based on the percentage of brown beans, was observed with the 10⁵ cells/g treatment. This supports previous findings that higher microbial concentrations enhance fermentation efficiency and shorten processing time (Gunama et al., 2021; Misnawi et al., 2017).

In parallel, the evolution of reducing and total sugars provided valuable biochemical insights into the fermentation process. Reducing sugars in the pulp sharply declined within the first 24 hours, from approximately 0.11 g/g to less than 0.02 g/g, across all treatments. This is consistent with the findings of (Reineccius et al., 1972), who reported substantial sugar degradation during fermentation. These reducing sugars, primarily resulting from the hydrolysis of sucrose via invertase activity (Afoakwa, 2016; Rohan and Stewart, 1967) serve as critical substrates for yeast and bacteria. They are also key precursors in the Maillard reaction, which produces flavor compounds during the drying and roasting phases (Afoakwa et al., 2013).

Interestingly, reducing sugar levels in the cotyledons increased progressively during fermentation, peaking at 0.12 g/g at 144 h for the 10⁵ cells/g treatment. This indicates both sugar diffusion from the pulp and internal enzymatic activity within the seed. The sugar accumulation in the cotyledons underscores the role of *C. tropicalis* in enhancing internal bean biochemistry.

The pH evolution during fermentation further corroborates metabolic activity. In the pulp, pH increased significantly over time, likely due to the degradation of citric acid by yeasts and Bacillus spp., which oxidize citric acid into ethanol and other metabolites (Van de Voorde et al., 2023). In contrast, the cotyledon pH decreased steadily, supporting previous reports by Apriyanto and Umanailo (2019). This pH decline is attributed to the migration of organic acids such as acetic and lactic acid from the pulp into the cotyledons.

Regarding total sugars, a significant reduction was observed in the pulp, dropping from 0.52 g/g to <0.10 g/g after 72 hours, indicative of active microbial fermentation and sugar conversion into ethanol, acids, and CO₂. This trend aligns with prior findings that non-reducing sugars and overall sugar content decrease significantly during fermentation (Afoakwa et al., 2013; Hashim et al., 1998). Conversely, total sugar content in the cotyledons slightly increased especially with the 10⁵ cells/g inoculum, suggesting efficient sugar transfer from the pulp to the seed.

Finally, the observed changes in sugar composition are closely linked to pH variations in the fermenting mass. As noted by (Peña González et al., 2023), the reduction of volatile acids and leaching of citric acid elevate the pH, facilitating enzymatic pathways that release fermentable sugars.

4. Conclusion

This study highlights the potential of *C. tropicalis* as an effective starter culture for controlled cocoa fermentation. The results clearly demonstrate that inoculation with *C. tropicalis* significantly enhances fermentation efficiency by improving bean quality and reducing fermentation time from six to three days using inoculum of 10⁵ and 10⁶ cells/g. These findings suggest that microbial inoculation could be a viable strategy to accelerate cocoa processing while ensuring consistent quality. Future research should focus on optimizing inoculum concentrations and refining fermentation conditions to maximize benefits for cocoa farmers while preserving desirable sensory attributes in the final product.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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