Study of Fermented Cacao Beans using *Aspergillus oryzae* Isolated from Pea Coffee Leaves

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ABSTRACT

Background: Chocolate is commercially made from the nut of *Theobroma cacao*. It has a bitter nutty taste, therefore many additives such as sucrose, milk, butter, etc are added to improve its taste and quality. The processing of cacao is an important step in the production of chocolate. It includes the fermentation of cacao beans using various microorganisms. In the present study, *Aspergillus oryzae* isolated from pea coffee leaves was characterised and was used to ferment cacao beans and the resulting change in its properties was studied.

Methods: In this study, we isolated *Aspergillus oryzae* from pea coffee leaves, using cooked rice medium. The isolated *Aspergillus oryzae* was then morphologically characterized, confirmed using molecular techniques by PCR-based amplification of ITS genes and was used to ferment cacao beans, to improve its flavour and quality. The changes in the flavour and other traits/attributes such as reducing sugar, protein, polyphenols, calcium, potassium and iron content of cacao beans before and after fermentation were estimated biochemically. The results were then compared with those of unfermented cacao beans and those fermented with yeast.

Result: The colour, smell, pH and taste of fermented cacao beans have considerably changed after fermentation. Quantitative analysis revealed that the contents of sugar, protein and polyphenols have decreased, while those of nutrients such as iron, calcium and potassium increased after fermentation.

Key words: *Aspergillus oryzae, Cacao*, Chocolate, Fermentation, Flavor, *Theobroma,* Yeast.

INTRODUCTION

For centuries chocolate has been used as sweet and savoury. Chocolate is made from cacao beans. The ripened cacao beans are harvested and their sweet, chewy flesh is removed using various means (including fermentation). These cacao beans are then dried under the sun or using a dryer. The beans then will be roasted, de-shelled, ground into a paste, sweetened and finally moulded into a bar of chocolate (Bariši *et al*., 2019). Cacao was used as a medicine in medieval times, due to its bitter flavour. This bitter flavour of cacao beans is due to the presence of different alkaloids and polyphenols (Stark *et al*., 2006). The bitterness of cacao beans can be reduced through fermentation while making a chocolate bar.

Due to the growth of microbes in the cacao beans during fermentation, they undergo certain biochemical and morphological changes (Lima *et al*., 2011). These changes will enhance the flavor of chocolate by reducing the unpleasant bitter flavour and mouth-drying astringency. Traditionally, the fermentation of cacao beans is done in a wooden box or banana leaf, with the help of various microorganisms. The fermentation of cacao beans can be carried out using several fungi such as yeast, *Aspergillus oryzae,* etc. The duration of fermentation will vary depending on the variety of cacao beans (Clapperton *et al*., 2021). For example, the Criollo cacao bean needs 2 to 3 days for fermentation whereas Forastero and Trinitario cacao beans require 5 to 7 days (Wahyuni *et al*., 2021). *A. oryzae* (Daba *et al*., 2021) and yeast (Maicas, 2020) have been used in various fermented foods for a very long period. *A. oryzae* can be isolated from rice, soybean, wheat, corn pods, coffee leaves, *etc*. (Chaves *et al*., 2012).

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The present study focuses on the isolation and characterization of *A. oryzae* from pea coffee leaves, the fermentation of cacao beans using this strain and its comparison with that fermented with baker's yeast.

MATERIALS AND METHODS

Isolation and morphological identification of *A. oryzae* from cacao beans

Fresh cacao beans and pea coffee leaves used in the present study were collected from Ernakulam, Kochi, India. The *A. oryzae* was isolated from the underside of pea coffee leaves using cooked rice medium, following the standard protocols described elsewhere (Vega *et al*., 2010). The cooked rice medium (Rice koji) was prepared by soaking rice in water until the weight increased by 30%, steam cooked until soft and the excess water if any was drained and the medium was transferred to a petridish (Yazui *et al*., 2020).

The coffee leaves were placed on top of the cooked rice medium, in such a way that the underside of the coffee leaves was in contact with the rice medium. The setup was incubated at 35°C for 3 days in a clean area. After incubation the petriplates were checked for fungal growth and the characteristic colonies with yellow-green colour were picked up and subcultured on fresh cooked rice medium. The optimal growth temperature for *A. oryzae* is 32°C-36°C but unable to grow above 44°C. Later the fungal colony was sub-cultured on Potato Dextrose Agar (PDA) medium. *A. oryzae* has characteristic morphological features such as white-coloured vegetative hyphae, which later produce yellowish-green conidia which turn brownish-green as it becomes old (Chaves *et al*., 2012). Upon microscopic examination *A. oryzae* exhibit typical morphological characteristics such as phialides, globose conidial heads and large 5-8 μm diameter spherical to slightly oval conidia (Daba *et al*., 2021).

Genomic DNA extraction

Approximately 0.01 g of the morphologically typical mycelium was ground with a mini grinder using 75 μL of STE extraction buffer (320 mM Sucrose, 10 mM Tris-Cl, 20 mM EDTA, 75 mM NaCl and 2.5 ml of 20% SDS) along with 5 mg of polyvinyl pyrrolidone and 0.1 g of silica powder and incubated at 65°C for 10 minutes. The sample was centrifuged at 13,000 rpm for 10 minutes. An equal volume of chloroform: isoamyl alcohol mixture was added to the supernatant and centrifugation was repeated. 2/3 volume of isopropanol was added to the aqueous layer and it was centrifuged at 13,000 rpm for 10 min. The pellet was washed with 70% ethanol, centrifuged, dried and dissolved in 50 μL TE buffer.

Molecular identification of *A. oryzae* using PCR

Molecular confirmation of *A. oryzae* was performed by PCR-based amplification of Internal Transcribed Spacer (ITS) region genes using the universal primers ITS1-5′ TCCGTAGGTGAACCTGCGG 3′ and ITS4-5′CAGGAGACTT GTACACGGTCCAG 3′. PCR amplification was performed in a total reaction volume of 25 μL consisting of sterile de-ionized water 18.7 μL, Taq buffer with MgCl2 (2.5 μL, 10 X), Forward Primer (0.5 μL, 10 pM/μL), Reverse Primer (0.5 μL, 10 pM/μL), dNTPs mix (0.5 μL, 10 mM each), Taq DNA Polymerase (0.3 μL, 5U/μL) and template DNA (2.0 μL, 25 ng/μL). Thermal cycler conditions included an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 45s and a final extension at 72°C for 10 min.

PCR product analysis by Agarose Electrophoresis, Gel documentation and sequencing

Electrophoretic separation of the PCR amplified products was performed on an agarose (1.5% w/v) gel in 1XTBE buffer (Himedia, India) containing 0.5 μg/ml of ethidium bromide and the amplicon sizes were compared with a 100 bp DNA ladder. The gels were then visualized and images recorded using the Gel Documentation System (GelDoc EZ imager, Bio-Rad, USA).

Amplified ITS gene segment was purified using column purification as per manufacturer guidelines (Thermo Scientific USA) and was subjected to automated DNA sequencing using ABI3730xl Genetic Analyzer (Applied Biosystems, USA). The nucleic acid sequences obtained were edited to correct falsely identified bases and used for similarity searches using Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov), NCBI to determine the percentage similarity with already identified ITS sequences in the GenBank DNA database for identifying the fungal strains.

Fermentation of cacao beans

The fermentation of cacao beans was carried out using the procedure described by Nurhayati and Apriyanto (2021) with slight modifications. The fresh cacao beans were added to *A. oryzae* cultured on cooked rice medium (Yasui *et al*., 2020) and fermentation was carried out for 3-7 days. After fermentation, the cocoa beans were dried under the sun, until the moisture was reduced to approximately 7%, which can be determined by cracking the epidermis of cacao beans (Nurhayati and Apriyanto, 2021).

Preparation of cacao extract

The cacao beans were taken out, roasted on a high flame, the outer cover removed, grounded using a mortar and pistil and subjected to biochemical analysis. Approximately 5 g of the powdered sample was added to 50 ml of distilled water and the mixture was kept in a boiling water bath for 10 minutes. The mixture was then cooled, filtered and stored at 4°C. (Castro *et al*., 2020).

Biochemical analysis

The sugar content of the cacao extract was checked using the Anthrone test (Yemm and Willis, 1954) and the total protein was estimated using the Biuret test (Janairo *et al*., 2015). Polyphenol content was determined by the Folin-Ciocalteau method (Ainsworth and Gillespie, 2007). The pH of the samples was checked using a pH meter (Sartorius, Germany). Changes in the nutrients such as iron, calcium and potassium of cacao beans after fermentation were tested using a flame photometer (Hald, 1946; Niedzielski *et al*., 2014).

RESULTS AND DISCUSSION

Fungal isolation, morphological and molecular identification

The fungal colonies exhibited typical morphological characteristics of *A. oryzae*. On potato dextrose agar medium, the fungal colonies appeared white with yellowish-green coloured spores which turned green with a brown shade upon aging (Fig 1). Upon microscopic examination phialides, globose to radiate conidial heads and large spherical to slightly oval conidia were observed

(Fig 2). Fungal DNA was isolated and observed as discrete bands on agarose gel using GelDoc EZ imager, Bio-Rad, USA. PCR amplification of ITS gene yielded amplicons of 1200 bp size, which was sequenced and upon BLAST analysis showed a 99.56% similarity with *A. oryzae* (NCBI Accession No: ON262681.1) with a query coverage of 100%.

Changes in physical properties of cacao beans after fermentation

Cacao beans after fermentation under various conditions showed differences in the physical properties as shown in Table 1. Changes in physical properties following fermentation have been reported previously (Romero-Cortes *et al.,* 2013; Sathe *et al*., 2016; Sujono *et al*., 2020). The fermented cacao beans have higher intense fruity, caramel, malty, flowery and toasty aromas than unfermented ones. The fermented cacao beans are sweeter, less astringent and bitter than unfermented ones (Cevallos *et al*., 2018; Schluter *et al*., 2022).

Biochemical analysis

Biochemical analysis revealed that the sugar content in unfermented cacao beans was 0.80 mg/ml, while that in cacao beans fermented with *A. oryzae* and yeast were 0.24 mg/ml and 0.28 mg/ml respectively (Table 2). The results indicated that the sugar content was lowest in the cacao beans fermented with *A. oryzae*. In the present study, the total sugar of fermented beans with *A. oryzae* has been reduced by 70%. A similar study by Santos *et al*. (2020) reported a 55% reduction in the total sugar content. Several other studies have reported a similar reduction in sugar after fermentation (Afoakwa *et al*., 2011; Calvo *et al*., 2021). During fermentation, the sugar present in the pulp will be converted into organic acids by microorganisms, which will induce enzymatic reactions inside the cacao beans that will result in biochemical changes inside the cacao beans generating certain compounds that will give better taste, color and aroma for the cacao beans. These continuous biochemical/chemical changes that happen over time would lead to changes in the biochemical contents of the cacao bean (Apriyanto, 2016; Puerto *et al*., 2016).

In the present study, a reduction in the total protein content of the cacao beans was observed after fermentation. The protein content in unfermented cacao beans was estimated to be 0.56 mg/ml, whereas that in cacao beans after fermentation with *A. oryzae* and yeast was reduced to 0.42 mg/ml and 0.43 mg/ml respectively. The results indicated that the amount of protein decreased considerably following fermentation and it was lowest in the cacao beans fermented with *A. oryzae.* In concordant with our results, several other studies have reported a similar reduction in protein content after fermentation (Romero-Cortes *et al*., 2013; Afoakwa *et al*., 2011). The decrease in protein content is due to the action of proteases in the cacao seeds which will hydrolyse the proteins to amino acids leading to an increase in the peptide concentration and the amount of amino acids. The free amino acids produced during fermentation will help to develop a nutty, chocolaty aroma during the heating process. The organic acids produced during fermentation will leach into the seeds of cacao and lead to a rise in the temperature that will result in the degradation of cacao tissue by decomposing proteins to the flavor precursor (Fang *et al*., 2020).

Polyphenols are one of the major factors defining the quality of the cacao bean used for chocolate production. In the present study, the total phenols in unfermented cacao were estimated to be 11.35 mg/ml, 7.43 mg/ml in cacao beans fermented with *A. oryzae* and 8.50 mg/ml in those fermented with yeast. Reduction in the total phenol content of cacao beans fermented with *A. oryzae* has been previously reported (Romero-Cortes *et al*., 2013; Calvo *et al*., 2021). In the present study, approximately a 35% decrease in polyphenolic content has been observed after fermentation with *A. oryzae*. Previous studies have reported a drop in total polyphenols to about 20% (Romero-Cortes *et al*., 2013; Calvo *et al*., 2021). A decrease in polyphenols improves the property of cacao by reducing the astringency (Afoakwa *et al*., 2012).

Variation in the pH was observed during the fermentation study. It is assumed to be due to the infiltration of acetic

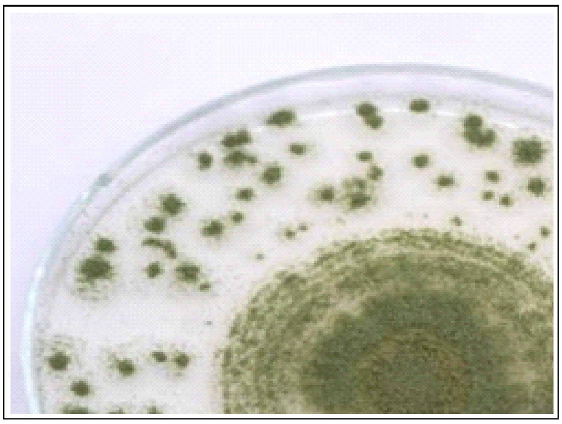

Fig 1: Colonies of *Aspergillus oryzae* isolated from pea coffee leaves after 5 days of incubation.

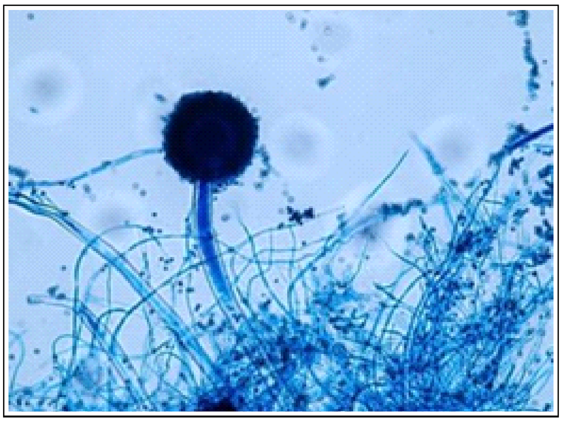

Fig 2: Microscopic view of *Aspergillus oryzae.*

Table 1: Comparison of changes in physical properties of cacao beans before and after fermentation.

	Smell	Taste	Color
Unfermented	Nutty	Nutty, astringent	Syrup color
A. oryzae	Milky	Milky	Penny color
Yeast	Pungent- Yeasty	Yeasty	Brown color

Table 2: Biochemical and nutrient changes observed in cacao beans before and after fermentation.

Biochemicals	Cacao extract		
	Unfermented	*A.oryzae*	Yeast
Sugar (mg/ml)	0.80	0.24	0.28
Polyphenol (mg/ml)	11.35	7.43	8.5
Protein (mg/ml)	0.56	0.42	0.43
Calcium (mg/ml)	0.1	0.2	0.2
Potassium (mg/ml)	4.9	9.1	4.6
Iron (mg/ml)	0.05	0.21	0.07
pH	6.45	5.89	6.87

acid produced during fermentation into the cacao beans since the bean pulp is permeable to acetic acid. This leads to the cellular death of cacao beans, killing the embryo and lowering the pH to 4.8 (Puerto *et al*., 2016). The pH of unfermented cacao beans was found to be 6.45, while that of cacao beans fermented with *A. oryzae* was 5.89 and that fermented with yeast was 6.87. The pH of fermented cacao beans with *A. oryzae* varied greatly from unfermented and fermented cacao beans with yeast. A similar decrease in pH was observed in previous studies (Afoakwa *et al*., 2011; Apriyanto *et al*., 2016; Santos *et al*., 2020; Calvo *et al*., 2021). In the study by Santos *et al*. (2020), the final pH of the cacao beans had reached up to 6. Van der Schueren *et al*. (2020) also reported a pH decrease of 2%.

Nutrient analysis showed that the levels of calcium, potassium and iron in unfermented cacao were 0.1 mg/ml, 4.9mg/ml and 0.048mg/ml respectively. In cacao beans fermented with *A. oryzae* 0.2 mg/ml, 9.1 mg/ml and 0.219 mg/ml of calcium, potassium and iron were observed respectively. The amount of calcium, potassium and iron in fermented cacao beans with yeast was found to be 0.2 mg/ml, 4.6 mg/ml and 0.06541 mg/ml respectively. The results indicate that the amount of nutrients considerably increased in the cacao fermented with *A. oryzae*. The concentration of calcium, iron and potassium has increased in fermented cacao beans considerably. Previous studies have also reported a similar increase in the mineral content of cacao beans followed by fermentation (Romero-Cortes *et al*., 2013).

CONCLUSION

The flavour quality of chocolate usually depends on the sources, processing and variety of cacao beans. Fermentation is one of the main steps in the processing of chocolate. The microbial flora used for fermentation significantly influences the flavour of chocolate produced. It was also observed during this study that the mineral compounds in the cacao beans improved considerably. The traditional cacao fermentation is carried out using a mixture of various organisms. In the current study, a comparison of cacao fermentation with *A. oryzae* and yeast was carried out, proving that the quality and flavour of the cacao beans will vary according to the organism used for fermentation. The flavour of chocolate made from fermented cacao using *A. oryzae* was found to be less astringent and creamier than that made using yeast and unfermented cacao beans. Thus, it is concluded that *A. oryzae*, which is used in traditional Asian fermentation is an ideal organism that can be used for cacao bean fermentation.

Conflict of interest

The authors disclose that there is no potential conflict of interest.

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