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Isolation, identification and characterization of yeast species from coffee waste collected from Sidama and Gedio zone

Birhanu Gizaw*, Zerihun Tsegaye and Genene Tefera

Microbial Biodiversity Directorate, Ethiopian Biodiversity institute, P. O. Box 30726 Addis Ababa, Ethiopia.

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Coffee waste represents the most abundant waste in Ethiopia, this study aims to isolate, identify and characterize yeast from coffee waste in order to utilize in the biotechnological process. 25 merged coffee waste samples were collected from Sidama and Gedio zone. Yeast strain was cultured on yeast peptone dextrose and Biolog universal yeast agar media. Pure yeast cells were suspended in sterile water at 49+ turbidity, 100 µ L and transferred into 96 wells of the biolog yeast micro Plate and incubated at 26°C for 24 to 72 h and read by the Micro Station Reader at a single wavelength of 590 nm, results were recorded and processed for identification by micro log3 software ver. 4.20.05. Biolog microstation acceptable result ≥ 75% Probability and ≥ 0.5 similarity index value identified 5 yeast species, *Hanseiaspora valbyensis*, (100%PROB,0.707SIM,), *Hyphopichia burtonii* A (98%PROB,0.060SIM), *Rhodotorula hylophila* (98%PROB, 0.060SIM), *Rhodotrula aurantiaca* A (100% PROB, 0.505SIM) and *Pichia amenthionina var. menthionina*, (PROB 96% SIM,0.714). There was no report on yeasts associated with coffee waste in Ethiopia for utilization in biotechnological process. Therefore characterization of yeasts is very important for industrial and environmental application.

Key words: Biolog, coffee, fermentation, micro station, Omni log, tetrazolium.

INTRODUCTION

Coffee is indigenous to Ethiopia and produced in large scale (Mutua, 2000). Coffee production is mainly in South west, South and East of the country. Of the estimated 600,000 ha of land cropped with coffee, it can be classified as: (i) Garden coffee (50%); (ii) Semi-forest coffee (35%); (iii) Forest coffee (10%); and (iv) Plantation coffee (5%) (Aga et al., 2003). Coffee waste represents the most abundant and non-edible agricultural waste obtained by wet process from red cherries of coffee. Thus, for every 2 tons of coffee cherries processed, nearly one ton pulp is generated. It represents about 40% of the fruit on a fresh weight basis (Adams and Dougan, 1981) and 29% on a dry weight basis (Bressani, 1979). The coffee bean (endosperm) represents about 45% of the fruit; the other 55% is generally discarded as waste (pulp and mucilage). It has been calculated that nearly an

*Corresponding author. E-mail: gbirhanu@ibc.gov.et.

availability of 525,000 tons per year of coffee residue has been estimated in Ethiopia, obtained from wet coffee milling (Alemayehu et al., 2007). And this huge agro industrial waste is disposed off in the environment without any remarkable use and considered to be the major polluting agent of the environment located near the coffee-processing regions. Coffee fruit pulp and mucilage consist primarily of water (76%), protein (10%), fiber (21%) and minerals (8%). The remaining 4% is composed of different types of soluble and insoluble matter (pectin, tannins, reducing and non-reducing sugars, caffeine, chlorogenic and caffeic acid, cellulose, hemicelluloses, lignin and amino acids). The presence of these proteins, sugars and many other nutrients are easily available for a wide variety of microorganisms (Silva et al., 2010). The natural coffee fermentation process involves a diversity of yeasts (Silva et al., 2000) and filamentous fungi (Avallone et al., 2001; Masoud et al., 2004). Several studies have isolated yeasts from natural fermentation of Robusta coffee bean includes *Kluyveromyces marxianus*, *Saccharomyces bayanus, Saccharomyces cerevisiae* var. *ellipsoideus* and *Schizosaccharomyces* species in India (Agate and Bhat, 1966). In Brazil coffee fermentation environment, *Debaromyces hansenii*, *Pichia guilliermondii*, *Pichia burtonii, Debaromyces polymorphus, Arxula adeninivorans, Pichia holstii, Pichia anomala* and Candida species were isolated (Silva et al., 2008). *Pichia fermentans, Pichia kluyveri, Candida glabrata, Saccharomyces* specie*, Pichia guilliermondii, Pichia caribbica and Hansenia sporaopuntiae* are some of the most isolated yeast strains from coffee (Demelo et al., 2014). The lignocelluloses and starch rich substrates of coffee pulp are found to be potential biomass substrates for bioethanol production through microbial fermentation (Amelia et al., 2010). Several alternatives have been proposed for the utilization of coffee pulp, such as animal feed, organic fertilizer, or as a substrate for enzyme production (Antier et al., 1993) for biogas production, silage, edible mushroom production (Martinez, 1989). The study of economically significant yeast species from different substrate has increased interest in applications of biotechnology (Sheela et al., 2008), medical research (Rad et al., 2012) and biodiversity (Kurtzman and Robnett, 1998). Due to population growth in Ethiopia the demand of energy is increasing rapidly and huge annual budget is allocated for importing fuel, searching for an alternative energy source from lignocellulose waste by the use of microbial fermentation is crucial and timely important. There are 525,000 tons per year of coffee residue disposed to the environment without any use, in order to convert this lignculose waste into bio ethanol, the first Priority is given for identification and characterization of coffee waste natural micro flora for practical application. Even though there are many reports describing the composition, conservation, and up-grading utilization of coffee waste, there is no report on yeast species diversity associated in coffee waste for further utilization in the biotechnological process in Ethiopia. There for the main objective of this work was to identify and characterize the yeast strains from coffee waste that would have vital role in industrial and environmental application in production of value added bio products.

MATERIALS AND METHODS

Study area

The study was conducted in Gedio and Sidama zone in five selected districts, particularly in Yergachefee, yergalem, Aposto, wonago and Dilla zuria areas which is one of the 13 zones in south Nation, Nationalities, and People Regional States (SNNPR) in Ethiopia. The zone is located approximately 400 km far from Addis Ababa. Sidama has geographic coordinates of latitude, north: $5^0$45″ and $6^0$45″ and longitude, East, 38 and 39°. Elevation ranges from 1500 and 2500 m above sea level. Gedio zone is located north of Equator from 5° 53″N to 6° 27″N Latitude and from 38^0 8″ to 38° 30″ East, Longitude. The altitude ranges from 1500 to 3000 m. Sidama and Gedio Zone is producing 63,562 tons of coffees per year. This represents 63% of the SNNPR's output and 28% of Ethiopia's total output.

Sampling design and collection

5 potential coffee growing districts were selected from Gedio and Sidama zone. 5 coffee waste dumping site from each districts were selected according to the duration of waste, 1, 2, 3, 4 and 5 months. Coffee waste sample collected at 4 transect and 9 sampling point and merge together in one sample tube. A total of 25 merged samples from 25 dumping sites actively fermented and pungent smelling coffee pulp and mucilage juice waste were collected during April 08-28 /2014 (Figure 1). Samples were kept in ice box and transported to Addis Ababa in Microbial biodiversity directorate laboratory at Ethiopian biodiversity institute.

Screening and isolation of yeasts

From each variety of samples 1 g of sample was taken and diluted serially up to 10^6 ml. About 0.1 ml of serially diluted sample was transferred by swab through the streaking technique on yeast peptone dextrose agar media (YPDA). Isolates were subcultured twice until pure colony appeared for morphological identification. A single yeast colony was streaked to Biolog universal yeast agar (BUY agar plate, (60 g/1 L) and incubated for 48 h at 26ºCfor yeast micro plate (YTMicroplate) inoculums preparation. The yeast was identified according to their morphological characteristics and Biolog Micro Station Reading.

Morphological identification

The morphologic characteristics of the isolated yeasts were examined after growth on yeast peptone dextrose agar media and Biolog universal yeast agar media at 26°C for two days, its colony morphology, form, size, elevation, Margin/edge, colony color were observed using hand lens as well as its percentage frequency were recorded.

Identification of yeast species using biolog microstation

The Biolog Micro station system for yeast identification consists of

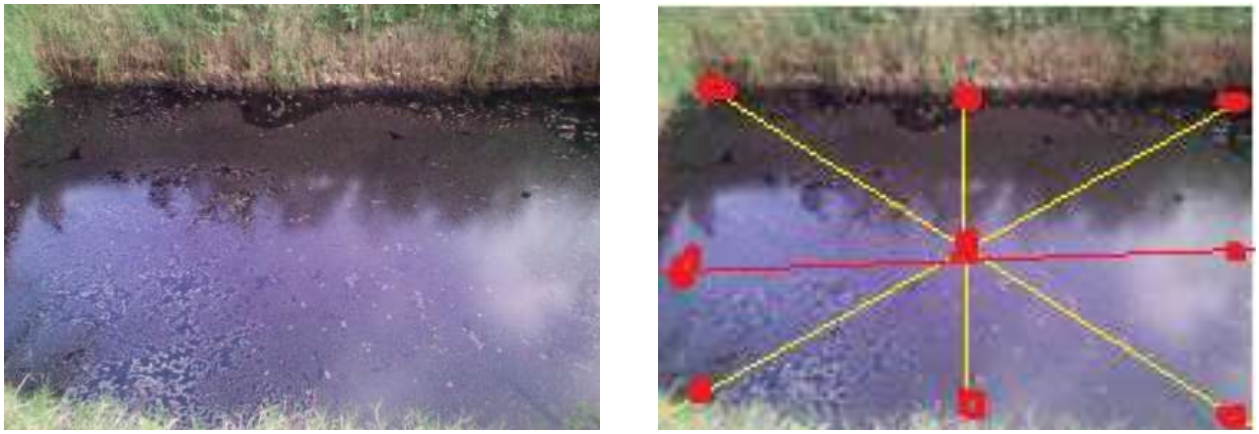

Figure 1. Coffee waste dumping site and sampling points.

Table 1. Percentage frequency yeast species on YPDA media (%).

Species	Number of colony	Percentage frequency on YPDA media (%)
Hyphopichia burtonii A	22	27.5
Rhodotrula aurantiac A	18	22.5
Hanseiaspora valbyensiskloecker	16	20
Rhodotorula hylophila	14	17.5
Pichiaamenthionina var amenthionina	10	12.5

micro plate tagged with 96 dehydrated carbon sources, a multichannel pipetter, a turbidiameter, a computer linked micro plate reader and Biolog Microlog3 softwarever. 4.20.05. Yeasts were subcultured to Biolog Universal Yeast Buy agar (BUY; BiologInc, Hayward, Calif., U.S.A.) and incubated at 26°C for 24 to 72 h. pure colony of yeast suspension was prepared in 9 ml sterile distilled water and adjusted to 47% T using Biolog YT turbidiameter. 100 µ L of inoculums was dispensed to each wells of the biolog yeast (YT) Micro Plate and incubated at 26°C. The YT Micro Plate measures both metabolic reactions as well as turbidity growth to produce identifications. A YT Micro Plate was read by the Micro Station Reader (BiologInc) at 24, 48 and 72 h at a single wavelength of 590 nm. The Biolog software micro log3 ver. 4.20.05 compared the results obtained with the test strain to the database and provided identification based on distance value of match and separation score produces similarity index value and probability. An acceptable species identification must have similarity index value ≥ 0.5 or probability ≥ 75% were chosen only for species identification and characterization. (Biolog, 1993).

Characterization of yeast using biology micro station assimilation and oxidation test

YT micro plate is configured with both oxidation tests and assimilation tests. The first 3 rows of the panel (rows A - C) contain carbon source oxidation tests using tetrazolium violet as a colorimetric indicator of oxidation. The next five rows of the panel (rows D - H) contain carbon source assimilation tests. Results from these tests are scored turbid metrically. The last row of the panel (row H) has wells that contain 2 carbon sources. These wells test for the co-utilization of various carbon sources with D-xylose. During incubation, yeast respiration in wells containing compounds that can be utilized will either reduce the tetrazolium dye forming a formazon purple color or initiate growth leading to an increase in turbidity. Each metabolic pattern was read by a Micro Station (Biolog Inc)) at a single wavelength of 590 nm and interpreted by micro log3 software ver. 4.20.05 (Biolog, Hayward, CA). Colorimetric or turbidity change in each well was referenced against negative control wells. Micro plate wells are scored as negative, (-), as positive <+>, borderline (\) for its carbon assimilation and oxidation.

RESULTS

Macro morphological characteristics

Eighty colonies were grown on YPDA media and observed under hand lens. Their percentage frequency on YPDA media was recorded, the result revealed *H. burtonii* A (27.5%), *R. aurantiac* A (22.5%), *H. valbyensis kloecker (*20%), *R. hylophila* (17.5%) and *P. amenthionina vara menthionina,* (12.5%) (Table 1).Their shape, size, elevation, margin and color of the colony were observed in the yeast peptone dextrose agar culture plates (Figure 2). Macro morphological results were noted down (Table 2).

Biolog microstation yeast identification

A similarity index was calculated based on the reaction profiles in 96 dehydrated carbon sources tagged on yeast

Figure 2. Isolated yeast strain and colony on YPDA media. A = *H. valbyensis kloecker;* B = *R. aurantiaca*A C = *R. hylophila.* D= *H. burtonii* A E=*P. amenthionina vara menthionina*

Table 2. Colonial morphology of yeast isolated from coffee pulp waste.

Yeast species	Colony chararacteristics						
	Shape	**Size**	**Elevation**	**Surface**	**Color**	**Texture**	**Marigin**
H. valbyensiskloecker	Circular	Medium	Raised	Smooth	White	Butrous	Round
R. aurantiacA	Ovoid	Medium	Raised	Rough	Red	Moist	Ring
R. hylophila	Circular	Medium	Raised	Rough	White	Moist	Entire
H. burtoniiA	Circular	Small	Raised	Smooth	White	Moist	Round
P. amenthionina varamenthionina	Circular	Medium	Raised	Smooth	Yellow	Moist	Entire

Table 3. BiologMicrostation yeast identification.

Status	Probability (%)	Similarity	Distance	Species
H. valbyensiskloecker	100	0.707	4.46	Identified
R. aurantica A	100	0.505	7.86	Identified
Hyphopichiaburtonii A	98	0.567	6.55	Identified
R. hylophilia	98	0.601	5.98	Identified
P. amenthionina var.*amenthionina*	96	0.714	2.80	Identified

micro plates for those selected and representative colony. At 24 h an acceptable species identification must have similarity index value 0.75 or above, and subsequent reading 48 to 72 h having similarity index value of 0.5 or above is needed (Biolog, 1993). By comparing with the yeast database (MicroLog TM System Release 4.2 User Guide 2001, Biolog), the result revealed that five yeast species were identified associated with coffee waste having≥75% probability and ≥0.5 similarities index value. The result summarized in Table 3.

Characterization of yeast species from carbon assimilation and oxidation test

Once the isolates are identified by Micro station, they were characterized in accordance with carbon assimilation and oxidation test. During incubation, yeast respiration in yeast micro plate wells containing tetrazolium compounds will either reduce the tetrazolium dye forming a formazon purple color or initiate growth leading to an increase in turbidity. Colorimetric or turbidity change in each well was referenced against negative control wells. Micro plate wells are scored as negative, (-), as positive <+>, borderline (\). The pattern is cross-referenced to a library of species in the database. The pattern of sugar assimilation and oxidation is given in Table 4.

DISCUSSION

So far about 1500 yeast species have been identified and they are ubiquitous in their distribution and populations largely depending on the type and concentration of organic materials. The distribution of species, as well as

Table 4. Yeast species assimilation and oxidation test.

Yeast species	Oxidize	Assimilate
H. valbyensis kloecker	AceticAcid, AsparticAcid, Melezitose, Palatinose, Turanose, Psicose.	Fumaric acid, Cellobiose, D-Melezitose, N-Acetyl-D-Glucosamine, α-D-Glucose,D-Psicose,L-Sorbose, β-Methyl-D-Glucoside,Arbutin,Mannitol, Adonitol,Glycerol, L-Arabinose,D-Arabinose, D-Ribose.
H. burtonii A	Proline, Succinic Acid, Aspartic Acid, GlutamicAcid, Cellobiose, Dextrin, GluconicAcid, Maltose, Maltotriose, Stachyose,Sucrose,α-D-Glucose, D-Galactose, D-Psicose,Salicin, Mannitol,Sorbitol.	Maltotriose, Maltose, Cellobiose.
R. hylophila	Aspartic Acid, Acetic Acid, Succinic Acid, Glutamic Acid, L-Proline,Mannitol, Glycerol,Tween 80.	L-MalicAcid, FumaricAcid, L-GlutamicAcid, 2- Keto-D-GluconicAcid.
R. aurantiaca A	Dextrin, Turanose , D-Trehalose.	Cellobiose, Maltose, Palatinose, Sucrose, Trehalose, Arbutin, Dextrinplus D-Xylose, D-Xylose.
P. amenthionina var. *amenthionina*	Propionic Acid	FumaricAcid, L-MalicAcid.

their numbers and metabolic characteristics were found to be governed by existing environmental conditions (Maragatham and Panneerselvam, 2011). Yeasts of the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, *Saccharomyces*, *Candida* and *Pichia,* amongst others, have been isolated mostly from fresh and rotten fruits (Fleet, 2003).

With the increasing interest concerning fermentation, industrial products issued from yeast biotechnology have emerged in many commercially important sectors, for instance, food, beverages, biofuels, chemical industrial enzymes, pharmaceuticals, and agriculture. Hence in this research isolation, identification and characterizing of yeast from coffee waste by using Biolog Microstation are very important as it will have a potential for industrial and environmental application in production of value aided bio products.

The percentage frequency of yeast species on yeast pepton dextrose agar media was calculated (Table 1). In these finding *Hypho pichia burtonii A* (27.5%), and *R. aurantiac A* (22.5%) were the dominant species. In Brazil coffee fermentation environment*, D. hansenii* was found in highest amount *(27%)* followed by *P. guilliermondii* (18.9%), *Candida* spp. (8%) and other yeasts such as *P. burtonii, D. polymorphus, Arxulaadeninivorans, Pichiaholstii and Pichiaanomala* (Silva et al., 2008).

This kind of difference in percentage frequency might be from sugar content of mucilaginous and pulp waste, the coffee species or the nature of dumping system allow more microbial colonization for carbohydrate degradation.

Biolog microstation identifies five yeast species *H. valbyensis klecker*, *R. aurantiaca* A, *H. burtonii* A, *Rhodotorula hylophila* and *P. amenthionina var. amenthionina* (Table 3). Demelo et al. (2014) reported that *Pichiafermentans, Pichiakluyveri, Candida glabrata, quercitrusa, Saccharomyces sp., Pichia guilliermondii, Pichiacaribbica and Hanseniasporaopuntiae* the most isolated yeast strain from coffee waste. Masoud et al. (2004) successfully identified yeasts involved in fermentation of *Coffee arabica* using D1/D2 LSU of 26S rDNA. They included *Pichia anomala, Pichia ohmeri, Pichiakluyveri, Hanseniaspora uvarum, Candida pseudo intermedia, Issatchenkia orientalis, Torulaspora delbrueckii and Kluyveromyces marxianus.* Several studies have isolated yeasts from natural fermentation of robusta coffee bean which includes *Kluyveromyces marxianus, Saccharomyces bayanus, Saccharomyces cerevisiae* var. *ellipsoideus* and *Schizo saccharomyces* species in India (Agate and Bhat, 1966). However the Pichia and Hanseniaspora Genera are identified and supported by other literature, all isolated yeast species from Sidama and Gedio zone coffee waste were new species except *Hypho P. burtonii* A this might be the nutrional profile of coffee pulp, type of coffee species, soil type or microenvironment of dumping site that will favors this yeast species.

Yeasts were characterized for their carbon utilization patterns (Table 4). *Hanseiaspora valbyensis* assimilate Fumaric Acid, Cellobiose, D-Melezitose, N-Acetyl-D-Glucosamine, α-D-Glucose, D-Psicose, L-Sorbose, β-Methyl-D-Glucoside, Arbutin, Mannitol, Adonitol, Glycerol, L-Arabinose, D-Arabinose, D-Ribose. Phaff (1970) reported that *H. valbyensis* assimiate Glucose and Glycerol. This is also reported by Beech and Davenport (1970) that *Hanseiasporavalbyensis* is a non saccaromyce yeast ferment simple sugar and usually present in the

initial phase of the fermentation of several apple musts. *H. valbyensis* is unusual yeast that is often found in traditional balsamic vinegar and cider fermentations. In this study *H. burtonii*A assimilates Maltotriose, Maltose, Cellobiose and oxidiz Proline, Succinic Acid, Aspartic Acid, Glutamic Acid, Cellobiose, Dextrin, Gluconic Acid, Maltose, Maltotriose, Stachyose, Sucrose, α-D-Glucose, D-Galactose, D-Psicose, Salicin, Mannitol, Sorbitol. Noel et al. (2014) sugesseted that *H. burtonii* A is positive assimilation for arabinos, galactose, ethanol, lactos, maltos, ethanol, starch, xylose and ferment sugars like glucose, galactose , maltose and starch. This is also supported by Boboye and Dayoowoyemi (2009). *H. burtonii* both ferment and assimilate Glucose, Galactos, Fructose, Mannose and assimilate only Sucrose, Lactose, Maltose Xylose and Arabinos. Takeuchi et al. (2006) suggested that *H. burtonii* has positive amylolytic activities Katos et al. (2007) sugessted that *H. burtonii* produce alpha amylas enzyme. Therefore *H. burtonii* would be good candidate yeast for coffee waste fermentation via acid hydrolysis into simple sugar or other fermentation activities. In this study *R. aurantiaca* A assimilates Cellobiose, Maltose, Palatinose, Sucrose, Trehalose, Arbutin, Dextrin plus D-Xylose, and D-Xylose. This also corresponds with the report of Frengova et al. (2003). Rhdotrula metabolize Glucose, sucrose and Manitol are considered to get positive assimilation. The yeast strain belonging to Candida, Pichia, Rhodotrula, and Yarowia genera are successfully used in biotechnology domains related to food and chemical industry, thuraptics and bioremediation based on their ability to assimilate and biodegrade different carbon source. Yeast species isolated from coffee waste *R. aurantiaca* A, *H. valbyensis*, *R. hylophila* and *P. amenthionina* var*. amenthionina* were assimilating the pentose and hexose sugar (Table 4). Bressani (1979) reported that Coffee pulp is essentially rich in carbohydrates, mainly composed of three groups of polymers, namely cellulose, hemicellulose, and lignin. Cellulose and hemicelluloses are sugar rich fractions of interest for use in fermentation processes, since microorganisms may use the sugars for growth and production of value added compounds such as ethanol, food additives, organic acids, enzymes, and others besides biofuels, several organic acids, including lactic, citric, acetic, and succinic acids, may be produced by cellulose conversion. The conversion of cellulose and hemicelluloses to hexose and pentose sugar like glucose, xylose, arabinose, mannose, may be subsequently converted to several products of interest such as ethanol (Mesa et al., 2010), butanol (Qureshi and Ezeji, 2008), hydrogen (Pan et al., 2010), organic acids (Mussatto et al., 2006), and hydroxyl methylfurfural.Xylose can also be used for the production of ethanol (Silva et al., 2010) as well as mannose and other hexose sugars (Jorgensen et al., 2010). In this study *R. hylophila*, *R. aurantiaca A Hanseiasporavalbyensis* and *H. burtonii*, can assimilate and ferment hexose and pentose sugar this will be a good ethanol producer from coffee pulp waste or other carbon source. This are supported by the work of Gellért et al. (2000) that yeasts, mostly non-Saccharomyces strains (Hanseniaspora/Kloeckera, Rhodotorula,Candida, Debaryomyces, Pichia) are most important to initiate wine fermentation. It has been reported that *H. valbyensis* is usually present in the initial phases of the fermentation of several apple musts (Beech and Davenport, 1970). However, the isolation and characterization of some dominant microorganisms from coffee waste it is still at infancy stage in Ethiopia. The ability of this five different yeast strains to metabolize sugars and convert them to ethanol was evaluated and compared in later studies.

Conclusion

1. All yeast isolated, identified and characterized from coffee waste were non-saccharomyces yeast.
2. Yeast isolated from coffee waste have an ability to assimilate hexose and pentose sugar which has economic importance and important for sugar fermentation that will lead to bio ethanol production.
3. Coffee waste is a good substrate for harboring of yeast species.

Recommendation

1. The results of this study indicate that the coffee waste has a complex community of yeasts with variable characteristics. Further studies are necessary to determine the role of the various yeast species in determining the overall alcoholic production.
2. In Ethiopia 77% of the total export earning for fuel importing, microbial conversion of coffee waste fermentable sugar into biofuel using yeast as an alternative energy source.
3. The non-Saccharomyces yeasts isolated from coffee waste recommended used for a mixed cultures in the overall quality of wine fermentation and alcohols industry is gate way to promising research.
4. Further studies are necessary to determine the role of the various yeast species yeasts isolated from coffee waste in determining the overall alcoholic production capacity and bakery industry.

Conflicts of Interests

The authors have not declared any conflict of interests.

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