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IDENTIFICATION OF BEER MICROFLORA THROUGH MICROBIOLOGICAL AND MOLECULAR BIOLOGICAL TECHNIQUES

Farwa Saeed[1], Muhammad Saud Javed[2], Ayesha kousar[3], Ateeqa Aslam[4], Dr Daniel Amund[5*]

[1]Faculty of Health and Life Sciences, Coventry University.farwasaeed222@gmail.com
[2]Department of Medicine (MBBS), Yangtze University, China. saudjaved1@hotmail.com
[3]Laboratory of Microbiology, department of translational physiology, epidemiology and public health, Merelbeke, Department of Biochemistry and Microbiology, Gent University, Belgium. Ayeshakousar981@gmail.com
[4]Laboratory of Virology, department of translational physiology, epidemiology and public health, Merelbeke, Department of Biochemistry and Microbiology, Ghent University Belgium. ateeqaaslam66@gmail.com
[5*]Faculty of Health and Life Sciences, Coventry University. ac1139@coventry.ac.uk

***Corresponding author:** Dr Daniel Amund
Faculty of Health and Life Sciences, Coventry University. ac1139@coventry.ac.uk

Abstract
Microbiological and molecular biological techniques play an important role in identification of the beer microflora. The purpose of the project is to identify and assessed the microbial strains of spontaneously ferment beer for their stability to brewing beer. On identification of suitable strain, the similar food-grade will be purchased to produce the approved food premises beer. This project concentrates on two major parts; one is microbiology-based identification of beer microflora which helps in detection of phenotypic traits of the microbial strains. Five industrial media (NA, M-R-S, VRGB, rose Bengal and universal beer agar) were used to evaluate the beer microbial strains. Second part describes the genotypic analysis of microbial strains through PCR, Big dye sequencing and NCBI-BLAST tool. From partial sequence of yeast isolates, *Pichia spp. Pichia occidentalis and Candida boidinii* is obtained which are non-conventional yeasts. With increasing demand of beer, these non-conventional yeasts are getting attention due to their unique flavour and aroma production. Overall, despite the poor sequencing data which shows ultimately that those species are present in the beer wort sample but due to partial sequence matching, the end results are ambiguous. If the sequences would have more complete, then a better mapped of more specific and reliable strains could be possible. Some limitations of the project are also discussed. This project also provides some suggestion to mitigate those limitations.

Keywords: Microbiological techniques, Molecular biological techniques, spontaneously fermented beer, Non-conventional yeasts, phenotypic traits, genotypic analysis

Introduction
Beer production is a complex fermentation process that has been practiced for millennia, driven primarily by the metabolic activity of yeast, particularly Saccharomyces cerevisiae (Suzuki, 2020).

During the brewing process, yeast ferments sugars derived from malted grains into ethanol and carbon dioxide, while also producing a range of secondary metabolites that contribute to the flavor, aroma, and mouthfeel of beer (Tsekouras, Tryfinopoulou, & Panagou, 2022; Xu et al., 2022). However, the microbial ecosystem involved in brewing is not limited to yeast alone; a diverse range of microorganisms, collectively referred to as beer microflora, can be present at various stages of production, from raw material handling to fermentation and packaging (Piraine, Leite, & Bochman, 2021).

Both useful and spoilage microorganisms are involved in the microflora of beers (Dysvik et al., 2020). Among them, many yeasts and bacteria are intentionally involved in specific styles, and their presence is important for the typical sensory features of traditional and craft beers (Dysvik et al., 2020). Brettanomyces species play a role in lambic and gueuze beers, adding desirable complications owing to the production of volatile esters and phenols (Kurniawan et al., 2021). The Lactobacillus and Pediococcus, are added to sour fermentations of beers to produce organic acids that give lactic acidity to the beer. In contrast, spoilage by undesirable microorganisms, including wild yeasts, spoilage bacteria, or mold, can result in product defects, such as off-flavors, haze, over-carbonation, and decreased shelf life. Lactobacillus and Pediococcus spp. might form acidification and turbidity, while Zymomonas may develop certain undesirable byproducts, including hydrogen sulfide (De Simone et al., 2021).

Identification of microflora is an utmost requirement for the brewing industry in terms of quality and consistency of the product (Ghesti, Carvalho, Carmo, & Suarez, 2023). Microbiological techniques based on culture have conventionally formed the core in microbial identification. The techniques involve isolation of microorganisms on a variety of selective or differential media, culturing them, and their characterization by colony morphology, biochemical activity, and growth conditions (Pauter, Szultka-Młyńska, & Buszewski, 2020). While very effective for the detection of many common beerspoilage organisms, culture based methods inherently cannot detect non-culturable or slow-growing organisms and are very labor and time-intensive (Romero-Rodríguez, Durán-Guerrero, Castro, Díaz, & Lasanta, 2022).

These challenges have been overcome by molecular biological techniques, which have emerged as powerful tools in microbial identification. Techniques including but not limited to PCR, qPCR, and 16S rRNA gene sequencing allow both the culturable and non-culturable microorganisms to be detected with rapidness, sensitivity, and specificity (Beyene, Tefera, Muleta, Fantahun, & Wessel, 2020). In assays based on PCR, particular DNA sequences that exclusively belong to target microorganisms can be amplified, hence enabling their identification even at low abundance. Besides, the NGS technologies give an overview of the whole microbial community within beer and are able to provide information concerning the diversity and dynamics of the beer microflora at a resolution not attainable by traditional approaches (Cason et al., 2020).

Aim of the project

The project aim is to analyse two spontaneously fermented beers which contain the local microflora. This project will look to identify the local microflora of the spontaneous beer through application of the microbiological and molecular biological techniques particularly using a Big Dye sequencing technique.

Methods and Materials

All the material was provided and funded by Coventry University. The project supervisors provided a bacterial DNA extraction kit, Universal beer agar, commercial beer, Rose Bengal agar (CM0115), and Nutrient agar (CM0003).

Preparation of wort samples

A sugar sample was made by boiling barley in water. Then, the boiled sample was split across six buckets and placed in fauna affluent areas across Coventry in the domestic garden. A muslin piece covered each bucket to stop any falling debris, animals or insects contaminating the liquid. The sample was placed under the following conditions mentioned in Table 1 below. Once the sample was received to process in the laboratory, it was stored at -80 ˚C.

Table 1: Sample No. date, time from, time to, location, description and weather of the six beer wort buckets (six wort buckets = six wort samples)

Sample No.	Time from	Time to	Location	Description	Weather
1	15:00	22:00	Waveley road, Coventry, CV1	Domestic garden, close to fauna	Mild, overcast, warm
2	15:00	22:00	Waveley road, Coventry, CV1	Domestic garden, close to fauna	Mild, overcast, warm
3	15:30	22:00	Osbaston Close, Coventry, CV	Domestic garden, close to fauna	Mild, overcast, warm
4	0:00	17:00	Billing road, Coventry, CV5	Near fruit trees on private allotment	Warm, sunny, humid
5	0:00	17:00	Billing road, Coventry, CV5	Under plum trees in shade on private allotment	Warm, sunny, humid
6	0:00	17:00	Billing road, Coventry, CV5	Open glade amongst grass, brambles and fruit bushes on private allotment	Warm, sunny, humid

Microbiological analysis

Preparation of sample dilution, culture media and enumeration

- Wort beer sample (stored in a universal vial) was taken out of -80 ˚C and kept at room temperature for 15 min (until it melts enough to withdraw 1000 µl of the sample). 10-fold wort sample dilution was made in normal saline solution.
- A dilution series was prepared from 10^{-1} till 10^{-7}. Then, 500 µl of the diluted sample from each of the dilution factor (10^{-1} to 10^{-7}) was spread on each of the agar plates;- Nutrient agar, Rose Bengal agar, Violet Red Bile Glucose (VRBG) agar (CM1082), De Man, Rogosa and Sharpe (M.R.S) agar (CM0361) and Universal Beer agar.
- Under the aseptic conditions (within 6 cm of the Bunsen burner) diluted sample was spread with the help of an aseptic spreader.
- After that, the plates were incubated as Nutrient agar (24- 48 hours), VRBG and universal Beer agar (3- 5 days) plates in an aerobic incubator at 37 ˚C. Whereas, M.R.S agar plates were stored in an anaerobic incubator at 37 ˚C for 3 to 5 days. Furthermore, the Rose Bengal agar plates were incubated at room temperature (25 ˚C) for 3 to 5 days.

Pure Cultures & Phenotypic characterization

After completing the incubation of each agar plate, the grown colonies of the bacteria and yeast were observed and characterized morphologically. From each media, every morphologically distinguished colony was purified on respective agar plates and stored under the same conditions mentioned above. To differentiate between purified bacteria and yeast isolates and to understand the type of bacteria (either gram-positive or gram-negative), gram staining was performed. The oxidase and catalase tests were also performed. For the oxidase test, OXIDASE (MB0266A) was used.

Molecular biological analysis

Overnight Cultures

Overnight culture of the purified yeast colonies (obtained from rose Bengal agar) was prepared in rich media. A single purified colony was picked from selective rose Bengal agar and thoroughly mixed (under aseptic conditions) in 2 ml of rich media YPD; yeast extract 1% (Bacto^{TM} Yeast Extract LOT 9024916), peptone 2% (Bacto^{TM} Peptone LOT 8312844), and Dextrose 2% (LP0071). Then, these cultures were incubated in shaking incubator at 30 ˚C for approximately 20 hours.

Bacterial Genomic DNA extraction:

Bacterial genomic DNA extraction was performed according to QIAamp® DNA mini Kit Cat. No. 51304 (All the buffers in the kit was ready to use).

- The only difference was in the first step in which each bacterial isolate was directly picked from the respective agar plate and was resuspend in 180 µl of ATL buffer and 20 µl of proteinase K. (ATL buffer and proteinase k.) was already present in the above mentioned bacterial genomic DNA kit), incubated in shaking water bath for 56 ˚C for 3 hours.
- After that 200 µl of AL buffer was added and vortex was done for 15 s. Again 10 min incubation was done at 70 ˚C (then to remove any drops from the lid, briefly centrifugation was performed). After that, 200 µl 100% ethanol was added in the same tube and vortexed for 15s.
- Then, the mixture was carefully transferred to 2 ml mini QIAamp column and centrifuged for 1 min at 8000 rmp. Collection tube containing flow-through was discarded and the column was placed into new collection tube (200 ml). 500 µl AW1 buffer was added to the column and centrifuged again for 1 min at 8000 rmp.
- After centrifuged the flow-through and collection tube was discarded. Then the column was put into a new collection tube and 500 µl AW2 buffer was added in it. The tube was then centrifuged for 3 min at 14,000 rmp. Again, the collection tube containing flow-through was discarded.
- Finally, the mini QIAamp column was placed into new (unprovided) 1.5 ml of collection tube and 200 µl of AE buffer was added in the column (incubated 5 mins at room temperature) to elute the DNA.

Isolation of Yeast Genomic DNA

For extraction of yeast genomic DNA, Harju, Fedosyuk and Peterson (2004) was used as a reference. From overnight cultures, the yeast cells were pelleted out by using microcentrifuge tube.

- Pellet was resuspended in 200 µl of lysis buffer (Triton X-100 2%, Sodium dodecyl sulfate (SDS) 1%, NaCl 100 mM, Tris-HCl (pH 8.0) 10 mM, Ethylenediaminetetraacetic acid (EDTA, pH 8.0) 1 mM). Then, this microcentrifuge tube was placed in -80 ˚C for 5 min (until the liquid completely frozen).
- After 5 min, the tubes were immediately thawed in 95 ˚C water bath for 2 mins. The tubes were vortexed for 30 secs. 200 µl of Phenol: Chloroform (1:1) were added in the tubes and vortexed for 2 mins and then centrifuged for 3 mins.
- From the two layers, the aqueous layer was carefully transferred to a new microcentrifuge tube containing 400 µl of the ice-cold 100% ethanol.
- The sample was allowed to precipitate for 5 mins and then the precipitated sample was centrifuged for 5 mins at 20000g at room temperature.

- The supernatant was vacuum evaporated through Pasteur pipette.
- The pellet was washed by using 70% ethanol (ice-cold). Then, the pellet was allowed to air dry for 5 mins. Finally, the pellet was resuspended in 30 µl of Tris EDTA (TE) buffer (10 mM Tris, 1 µl EDTA (pH 8.0). Concentration of the isolated genomic yeast DNA was measured through Nanodrop technology.

Bench Top Polymerase Chain Reaction (PCR)

PCR of the isolated genomic bacterial and yeast DNA was performed using universal primers. NS1 and NS4 universal primers were used to amplify the yeast genomic DNA, while 8F and 1492R universal primers were employed for the amplification of bacterial genomic DNA. The details of the universal primers are presented in Table 2.

Table 2: Universal primer names and primer sequence (obtained from the original primer sequencing vial) used in PCR.

Universal Primer Name	Primer Sequence
NS4	5'-CTTCCGTCAATTCCTTTAAG-3'
NS1	5'-GTAGTCATATGCTTGTCTC-3'
1492R	5'-CAATTGTTCCTCGTTAAG-3'
8F	5'-AGAGTTTGATCCTGGCTCAG-3'

Table 3: Cat. No. of the PCR ingredients

Ingredients	Cat. No.
5X Q5 Reaction buffer	B9004S
10 mM DNTPs	N0447L
Q5 High fidelity DNA polymerase	M0273X

PCR was run for 39 cycles. For yeast, 55 ˚C annealing temperature was used. Whereas, 53 ˚C annealing temperature was used for bacterial genomic DNA. Below Table 4 is presenting the PCR cyclic conditions used for amplification of the product.

Table 4: showing the PCR stages, temperature, time and no. of cycles used for bacterial and yeast genomic DNA amplification.

PCR Stages	Temperature	Time
Initial Denaturation	97 ˚C	5 mins
Denaturation	94 ˚C	1 min
Annealing	56 ˚C	1 min
Extension	72 ˚C	1 min
Repeat for 39 cycles		
Final Extension	72 ˚C	10 mins
Hold	4 ˚C	--

Gel Electrophoresis

PCR products was analyzed through gel electrophoresis.

- 1.5% agarose gel was used for gel electrophoresis which was prepared in Tris/Borate/EDTA buffer (TBE) and 2.5 µl of 1 X Gel red (Biotium cat. 41003-1) was also added in the gel (for reading results).
- TBE buffer contained 89 mM tris base, 89 mM boric acid, 2 mM EDTA.NA22H20 per 1 liter with pH 8.0. In casting tray (10 mm well), 10 µl of 1: 5 loading dye (amplicon mixture), (Thermo scientific, R0611 loading dye was used) and 7 µl of DNA ladder (as molecular size marker), (Thermoscientific cat. Sm1113) was loaded in 10 mm well of the gel and run in 50 µl of TBE buffer at 70 volts for 1 hr and 30 mins.

- Then, the image was visualized in transilluminator (Gel Doc^{TM} EZ imager: Bio Rad).

Single Step PCR: (Big Dye reaction mixture for DNA sequencing)

For the sequencing purpose the applied biosystem terminator Big Dye v3.1 kit was used. A reaction mixture for single step PCR was prepared with following ingredients.

- Big Dye Terminator Sequencing Mastermix 2 x (10 µl): (This mastermix contained 2 µl Ready Reaction mix (contained enzyme, 200 µM dNTP, fluorescent ddNTPs), 3 µL 10 X sequencing buffer, 0.05 units/µl of a Taq, 0.6 µM of each primer set, and 4.5 µl MilliQ water). 3 µl of original PCR amplified DNA product whose volume was adjusted up to 10 µl (by adding DNase free water) was used.
- Then, the total volume 20 µl, (10 µl of the reaction master mixture + 10 µl of adjusted amplified DNA product) was used for single step PCR. For PCR, the Eppendorf Mastercycler Gradient machine was used.

Table 5: Presenting the single step PCR cyclic conditions to carry out the DNA sequencing process.

Cyclic Stages	Temperature	Time
Initial Denaturation	96 ˚C	1 min
Repeat of 25 cycles	96 ˚C	10 secs
Annealing	50 ˚C	5 secs
Extension	60 ˚C	4 mins

Ethanol precipitation of big dye single step PCR sequencing reaction

- Continuing with the 20 µl of DNA sequencing reaction (after single step PCR amplification), 3 µl of sodium acetate and 2 µl of 125 mM of EDTA was added in the reaction. Then, centrifuge was done for 20 mins at 13000rmp.
- After a careful removement of the supernatant, 250 µl of the 70% ethanol was added in the same tube. Again, the 10 mins centrifuge was performed. At the end, the pellet was allowed to air dry. After air dry, 10 µl of formaldehyde (fumigation cabinet was used) was added in the pellet.
- Sample was added up in the reading plate (for sequence analysis) and was ready to read in the applied biosystem 3500 genetic analyzers.

Bioinformatic tool

After obtaining the sequencing files from the applied biosystem (sequence reading machine), the sequencing files were analysed by using the bioinformatic tool. The sequence of each isolate was pasted in nucleotide BLAST (in query sequence). Then, in program section, somewhat similar sequences (blastn) was chosen and finally, the sequence was blasted by pressing BLAST button.

Ethics approval

The ethics approval was granted by the Coventry University. The certificate number is P109991. After the approval of the ethics application, the project was officially started in the Coventry university laboratory.

Results

Microbiological Analysis

On completion of incubation period, microbial growth was found on nutrient agar, rose Bengal agar, and universal beer agar plates. Whereas, no growth was found on VRBG and MRS agar plates. Microbial colonies (from nutrient agar. universal beer agar rose Bengal agar) were calculated.

Table 6: The countable number of colonies obtained from three different agars and final CFU/mL.

Type of Media	CFU/mL
Nutrient agar	2.5×10^{8}
Universal beer agar	2.3×10^{4}
Rose Bengal agar	2.0×10^{6}

Each of the dilution plates (of three different types of agar) were observed. Nutrient agar, universal beer agar, and rose Bengal agar and their specific isolated colonies from each of the agar media, the code given to these isolated colonies, microbial type, other biochemical test's results and specific morphological characteristics of the microbes of the isolates are shown in the below **Table 7**.

Table 7: Isolation of bacterial and yeast colonies from their respective agars, the code assign to these colonies, type of micro-organism, results obtained after gram staining, Catalase test, Oxidase test and specific colony characteristics of the isolates.

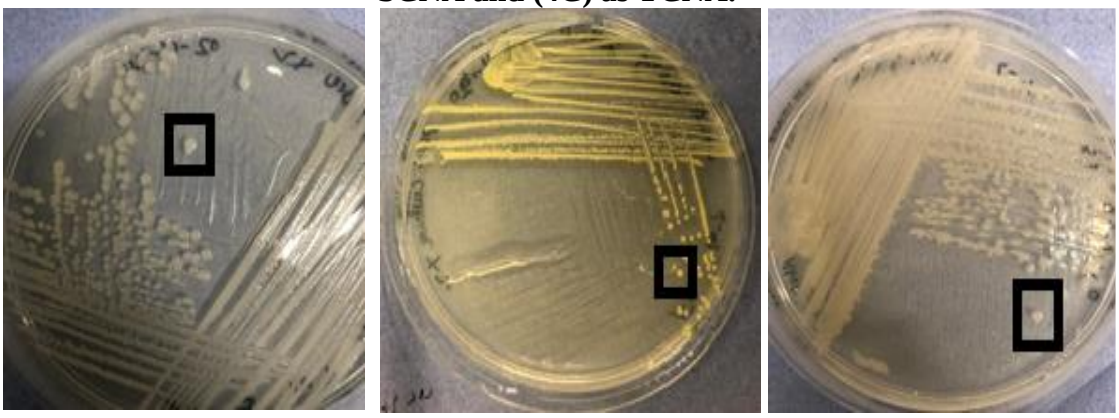
Media	Nameof isolated Colonies	Code assign to colonies	Bacteria (B) or Yeast (Y)	Gram Staining results	Catalase test (+/-)	Oxidase test (+/-)	specific colony characteristics of the isolates
Rose Bengal agar (RB)				-/+, Shape			
RB agar	White colony	WCRB	Y	Oval	+	+	White in color, raised from center, circular margin
RB agar	More pink colony	MPRB	Y	Oval	+	+	Dark pink and raised from center, circular margin
RB agar	Less pink colony	LPRB	Y	Oval	+	+	Less pink from center, raised and button in center, less pink and circular margin,
RB agar	Smallest pink colony	SPRB	Y	Oval	+	+	Smallest colony, pink color, raised from center, curly margin
Universal Beer agar (UB)							
UB agar	White small universal beer colony	WSUB	B	- , Rod	+	-	White, shiny, round margin

UB agar	Rough dry colony	RDUB	B	+, Rod	+	-	Rough, tiny button in center, flat, shiny, brown in color, irregular margin
UB agar	Smooth colony	SCUB	B	+, Rod,	+	-	Smooth, raised from center, dot in center, circular margin
UB agar	Button colony	BCUB	B	+, Rod	+	+	Big button with transparent margin, raised from center, circular margin
Nutrient agar (NA)							
NA	Small colony	SCNA	B	+, Cocci	+	+	Small, yellow, raised from center, circular margin
NA	Transparent colony	TCNA	B	-, Rod,	+	+	Transparent, flat colony, irregular margin
NA	Big colony	BCNA	B	+, Rod	-	+	Light yellow, raised from center, curly margin

Pure Microbial Colonies:

After the selection of differential morphological colonies, the pure isolates were obtained from plate culture method. On the nutrient agar plates, three morphologically different bacterial colonies were found.

Figure 1: The single pure colony morphology on nutrient agar after 48 hours of aerobic incubation at 37 ˚C (each plate refereed to a single type of colony). (4A) as BCNA code, (4B) as SCNA and (4C) as TCNA.

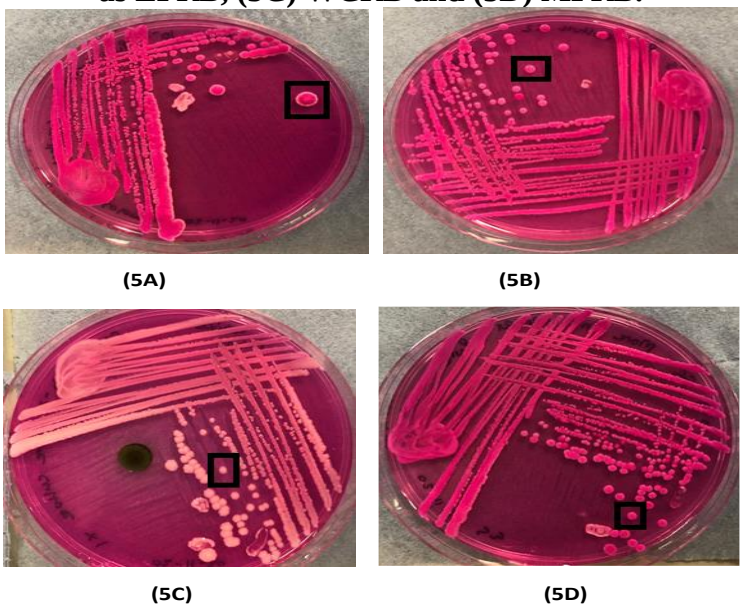


(4A) **(4B)** **(4C)**

Similarly, four different pure colonies on rose Bengal agar plate can be seen in Fig. 2 (5A, 5B, 5C, 5D). Rose Bengal agar was specific for yeast growth. No bacterial contamination was seen in the agar

(as chloramphenicol an antimicrobial was added during the rose Bengal agar preparation).

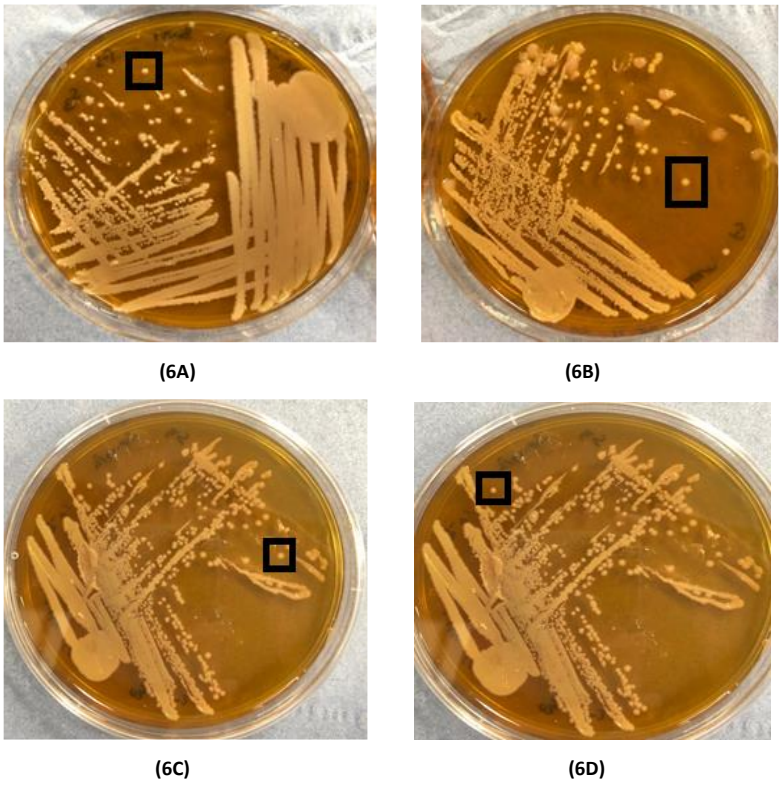
Figure 2: The single purified culture on rose Bengal agar after 5 days of aerobic incubation at 25 ◦C (each plate contains a different single type of colony). (5A) refereeing as SPK, (5B) code as LPRB, (5C) WCRB and (5D) MPRB.



Whereas four single isolates on beer agar plates are shown in Fig. 3 (6A, 6B, 6C, 6D). Each of below universal beer agar plate is presenting a morphologically different pure colony.

Figure 3. Showing a four universal beer agar plates. Each universal beer agar plate contains a different single type of isolate after 3 days of aerobic incubation at 37 ◦C. In these four agar plates, (6A) amed as WSUB, (6B) RDUB colony, (6C) BCUB and (6D) named as SCUB.

Biochemical identification of purified microbial colonies:



Further confirmation of the type of microflora was made by biochemical such as an oxidase test, catalase test, and gram staining of each respective purified colony. Rose Bengal agar is specific for the yeast growth. The result of gram staining showed that all the isolates obtained from rose Bengal agar have been confirmed as yeast. All the isolates obtained from NA agar were gram-positive except one colony which was transparent. Among the universal beer agar isolates, three were gram positive and one was gram negative. Besides, Table 7 gave the meaningful gram staining, catalase test and oxidase test of each pure colony.

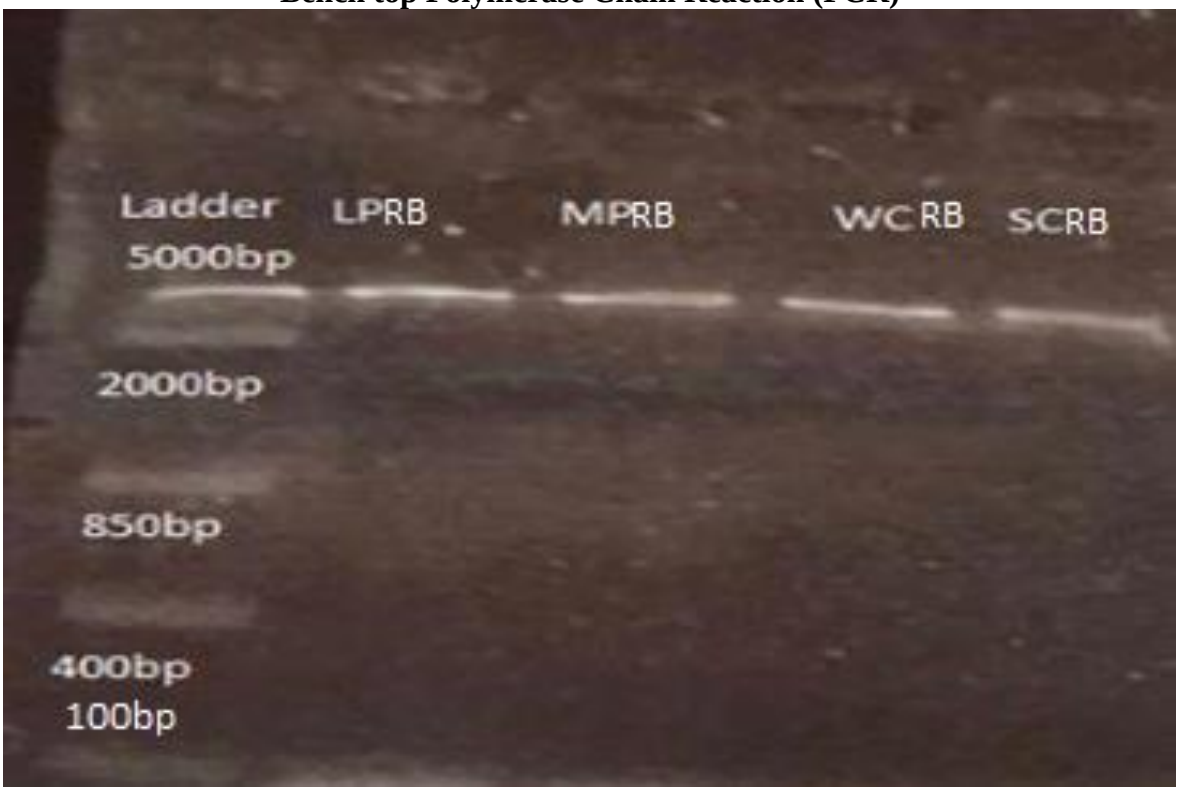
Molecular identification of Purified Microflora

Bacterial and Yeast DNA extraction:

The DNA was extracted from the purified bacterial and yeast colonies. Further, the yield and purity of the extracted genomic DNA of both bacteria and yeast were measured using a NanoDrop system. Results are shown in the appendix. In addition, the quality of the extracted yeast genomic DNA was checked with the help of gel electrophoresis.

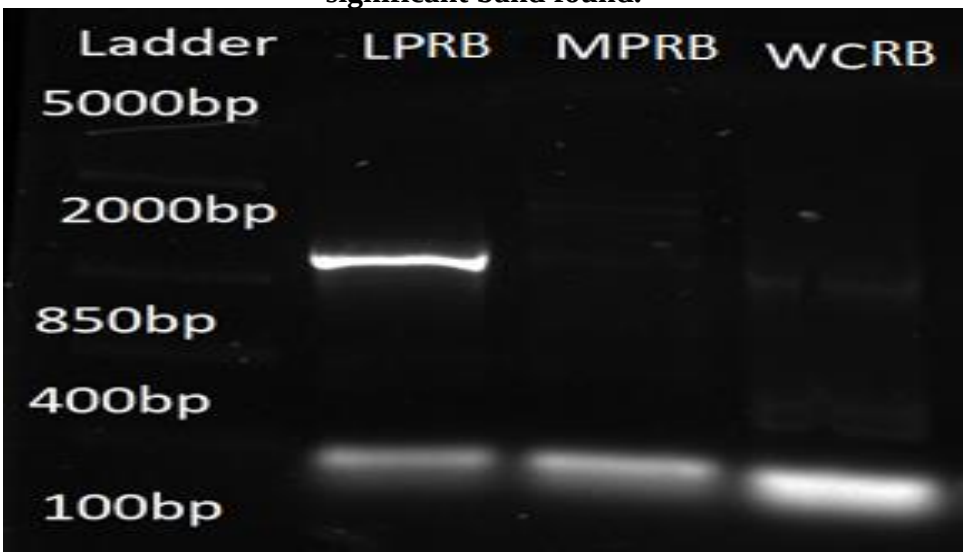
Figure 4: Gel electrophoresis results of four isolates of yeast from rose Bengal agar (yeast extracted genomic DNA). In each row, bands in accordance with 5000bp ladder, shows that the genomic DNA of the yeast was successfully extracted from the yeast cells.

Bench top Polymerase Chain Reaction (PCR)



Results of gel electrophoresis confirmed the amplification of the PCR product. The 18S rRNA gene was successfully amplified from the rose Bengal agar isolates. Fig. 8: The band closer to 1200bp is an indication that the PCR has worked successfully.

Fig 8: Genomic DNA of LPRB, MPRB and WCRB (all colonies belong to Rose Bengal agar: specific for the yeast growth) was used for this PCR. Ladder can be seen on the left side. Although, all the colonies were shown light band near about 1200bp. But a sharp clear band was seen for LPRB yeast colony at 1200bp. whereas for MPRB and WCRB isolates, less significant band found.



Sequence analysis using BLAST

The sequence of the amplified PCR product was evaluated using Big Dye sequencing, which was carried out through a series of reactions, including single-step PCR and ethanol precipitation. The sequence obtained from Big Dye sequencing was analyzed using the bioinformatics tool NCBI Nucleotide BLAST to identify any relationships to microorganisms. The sequence alignment obtained from NCBI Nucleotide BLAST is presented in Figures 9, 10, and 11.

Fig 9: The sequence alignment of the less pink isolate (LPRB) obtained from Rose Bengal agar showed low similarity to the *Pichia occidentalis* strain. This alignment indicated 155 nucleotide matches out of 210. Of the 55 anomalies, 48 were mismatches and 5 were deletions. The mismatched anomalies were highlighted in red within the alignment. The partial sequence for LPRB was provided in the appendix, labeled as LPRB-A01.

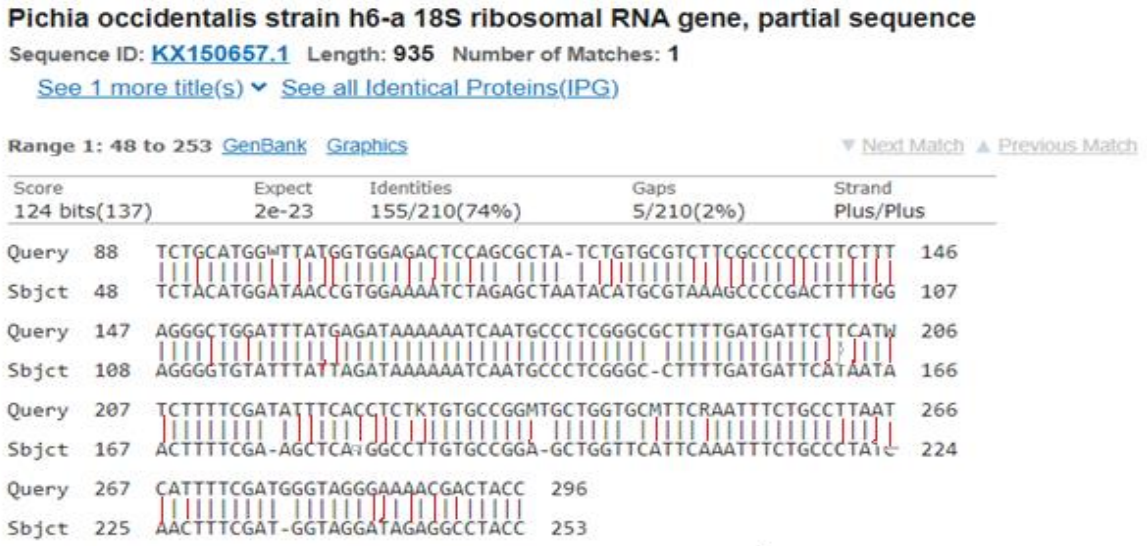


Fig 10: Another sequence alignment of the less pink isolate (LPRB) derived from Rose Bengal agar revealed a partial sequence matching to *Pichia sp*. The actual sequence is provided in the appendix as LPRB-B01. This alignment showed 351 nucleotide matches out of 506. Among the 155 anomalies, 15 were gaps, and 140 were mismatches.

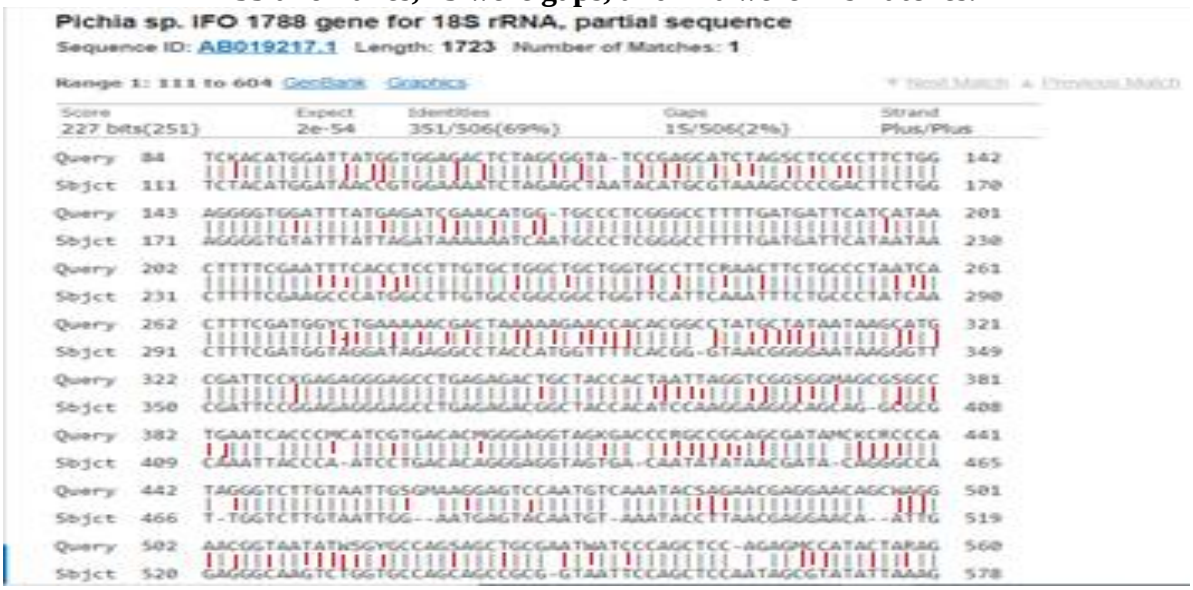
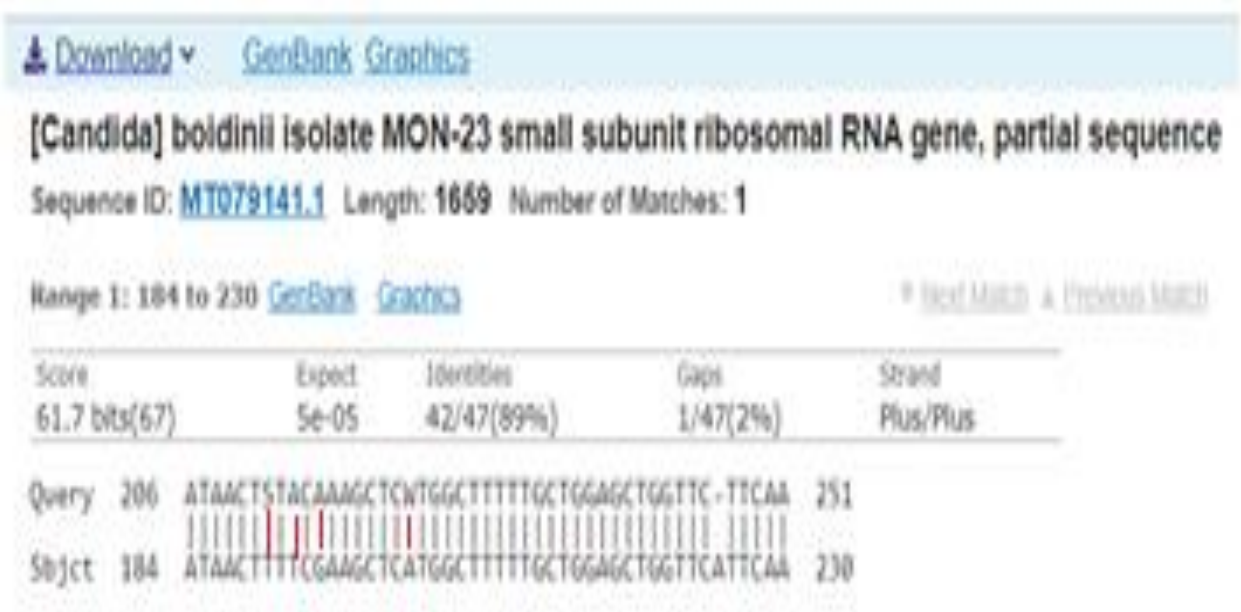


Fig 11: NCBI Nucleotide BLAST provided the sequence alignment for the white colony (WCRB) obtained from Rose Bengal agar, showing a match to the *Candida boidinii* strain. Out of 47 nucleotides, 42 aligned exactly. The mismatches are highlighted in red. Of the 5 anomalies, 1 was a deletion, and 4 were mismatches. The actual short sequence for the WCRB isolate is presented in the appendix as WCRB-F01.



Discussion

Microbiology based methods are used in the brewery industry due to cost effective and rapid detection of the beer microflora. Only a phenotypic trait of the microbe can be found through cultural methods. Cultivation, enumeration, observing the growth of microorganisms on selective agars with naked eye, colony counting are some suitable microbiological processes for detection. Some biochemical tests can also be used to narrow down the microbial research such as gram staining, catalase test, oxidase test and gas production analysis (Bossaert et al., 2021). Variation in the spread plate nutrient agar

results was found. As, with increase in dilution of original sample, the growth of microbes on the nutrient agar plates should be decreased. But a deviation was found in 10^{-1}, 10^{-4} and 10^{-5} dilution factor. The possible reason could be that the spreader was not properly aseptic after the spread of sample on previous diluted plate. Other expecting human error includes the problem during labelling of nutrient agar plates and formation of sample dilution. The colonies from respective agars were counted optically. From nutrient agar 2.5×10^{8} CFU/mL colonies, universal beer agar 2.3×10^{4} CFU/mL colonies and rose Bengal agar 2.0×10^{6} CFU/mL colonies found (shown in Table 6). Gram staining analysis helped to closely pack the beer microflora (as it supported in differentiation of microbes such as gram + bacteria, gram bacteria and yeast).

The gel electrophoresis results showed the intact and cleared bands at 5000bp, proved the quality of the extracted yeast genomic DNA. On gel electrophoresis, PCR product for yeast isolates have given a required band (near 1100bp). Band for LPRB isolate was sharp, intense and cleared. This sharp band showed that the 18S rRNA gene for LPRB isolate was successfully amplified. But the bands for MPRB and WCRB isolates were very fade and light. Although the faded bands were on the required position (1100bp). The reason of these light bands could be that the used amount of DNA for these isolates in PCR product is not enough. Another reason for these lighter bands could be intense primer-dimer formation. As some primer-dimers can also be seen in fig. 8 Primer-dimers can be defined as the interaction of primer-primer artefacts. They usually form due to off target amplification of primer-primer interaction. Therefore, it will interrupt the target DNA amplification (Luan et al., 2023). According to (Zugravu, Medar, Manolescu, & Constantin, 2023) the primer-primer interaction can stop target DNA amplification.

The big dye sequencing results. Before analysing the big dye sequencing results in the sequencing machine, ethanol precipitation of PCR product was done to remove any extra primer or fluorescent deoxyribonucleotide triphosphate (helped to avoid any interruption in the sequencing). From three PCR products, LPRB isolate gave two short but readable sequences. Whereas, WCRB isolate gave one short readable sequence and MPRB isolate did not provide any readable sequence. For LPRB and WCRB sequences were analysed on nucleotide BLAST. For LPRB both short sequences gave almost similar organism identification. One of the sequences (LPRB-A01) gave a specific strain "Pichia occidentalis" (shown in fig. 9) and other sequence (LPRB-B01) gave *"Pichia spp"* and maximum organism identity was 73.81%. Whereas, on WCRB (WCRB-F01 sequence) isolate's sequencing on the nucleotide BLAST gave a specific strain known as *"Candida boidinii"*. The maximum strain identity was 89.36%.

Candida boidinii is a yeast belongs to phylum *Ascomycota* and class *Saccharomycetes*. This yeast is non-motile, salt tolerant and aerobic (sometimes facultative anaerobe). The optimum temperature for *Candida boidinii* is 25 ˚C to 30 ˚C. *Candida boidinii* is a habitant of wine fermentation. In Spain, this yeast was firstly identified through washing of tree bark (Yu et al., 2021). *Pichia* another yeast genus which is characterized as hat shaped ascospores, pseudo hyphae may or may not be present and septate hyphae (Adesokan, Sanni, & Marc-Andre, 2020). *Candida spp*. and *Pichia spp*. both belongs to non-saccharomyces yeast (Ovalle-Marmolejo, Redondo-Solano, Granados-Chinchilla, Miranda-Castilleja, & Arvizu-Medrano, 2023).

According to (Ciont et al., 2022) *non-saccharomyces* yeast is considered dominant in earlier stages of spontaneous fermentation. Whereas, *saccharomyces* strains usually found to be prominent in later stages of beer spontaneous fermentation. Now a days, brewing industries use pure yeast starter culture for better fermentation process control but fail to produce unique flavour and aroma (Syrokou et al., 2020). Some studies showed that from past few decades, *non-saccharomyces* yeasts have been recognised for spoilage in beer, therefore, it got very little attention in the brewing industry. Now, with the increasing interest in craft brewery, these non-conventional yeasts may help to give a unique flavour and flavoured compounds (Adejobi et al., 2024). These yeasts also contribute in high ester

amounts. Due to the volatile flavour and compound properties of esters, they give additional aromas into the beer (Yang et al., 2022). Ethyl acetate, Phenylethyl acetate and ethyl decanoate are some of the esters which produce the fruity sensory expression (Petkova, Stefanova, Gotcheva, Kuzmanova, & Angelov, 2020). According to (Gholami-Shabani, Shams-Ghahfarokhi, & Razzaghi-Abyaneh, 2023) some of *Candida* and *Pichia spp.* produce honey aroma. *Pichia spp*. can also be characterised as solvent smell production (Bossaert et al., 2022). Moreover, during the late maturation phase of Lambic beer, it is found that a biofilm is formed by *Brettanomyces spp.* along with *Candida a*nd *Pichia spp*. which prevent beer oxidation. In barrel, this biofilm grows on top of liquid interface (Gerard, Davies, Soldá, Corrado, & Fernández, 2020).

Some studies showed that the presence of *Pichia spp*. and *Candida boidinii* is due to human contamination which may affect the organoleptic properties of the beer. Such as shortened the shelf life of the beer. Therefore, these beer products will complete the consumer demand for only short term. In brew-pub productions, no evident found for these problems. As the beer is usually not bottled, instead of that beer is directly served to the consumer within short period of their production. Moreover, no change in beer pH (normal range 4.0- 4.6) was found due to the contaminants (Deb, Das, Adak, & Khan, 2020).

Limitation and Areas of improvements

In this research the microbiological part was successfully completed. Although, culturally based methods provide quick identification, but a limitation have been seen with respect to spontaneous beer fermentation as only a few numbers of isolates can be identified by these methods. Recent studies on polyphasic taxonomy showed that the identification of the organisms at specie level is inadequate only through phenotypic identification methods (Sun et al., 2022). Due to time shortage (Covid-19 pandemic), bacterial isolates were processed till genomic DNA extraction level.

As the project was based on analysis of spontaneous beer fermentation microflora but there was no time to work on actual fermentation sample (only wort sample was processed). So, there is a possibility that most of the micro-organisms (belongs to spontaneous beer fermentation) have not been grown or maybe they do not have a detectable level yet.

Lack of time impacted on the quality of results. One of the main problems, Big Dye sequencing didn't work well, there was lots of unconfirmed nucleotides with the sequence, lead to low sequence similarity. To improve, Next gen sequencing (NGS) technology can used. NGS is cost effective (Bucka-Kolendo, Sokołowska, & Winiarczyk, 2020), deep and high throughput DNA sequencing analysis technique. It generates the giga base (Gb) in only few days to hours (Bazalová et al., 2022). Next generation sequencing has the ability of detailed analysis of single nucleotide variation (SNV), detection of deleted and duplicated sequences (Nejati, Junne, Kurreck, & Neubauer, 2020). Therefore, NGS could be an option sequence data analysis.

If there would be more time, then PCR could be optimised to avoid any primer dimer formation. Hot start PCR can be used to avoid primer dimer formation (Fayyaz et al., 2022). Other methods including reduction in primer concentration, increasing the PCR extension time, decreasing the PCR extension temperature and changing the no. of PCR cycles may help to minimise the primer dimer formation (Monica et al., 2021).

Overall, it was still good that some of the microbial species identified, despite the poor sequencing data which shows ultimately, that those species are present but, if the sequences would have more complete, it was possible to map it to a more specific strains.

Conclusion

From LPRB isolate of the rose Bengal agar, *Pichia spp.* specifically the *Pichia occidentalis* was found. As, the full sequence was not available to matched. Therefore, the results could not be 100% reliable.

For the WCRB which has matched to a small sections of *Candida boidinii.* Which isn't 100% inclusive. Therefore, the results for both isolates is highly ambiguous and required further analysis. Due to the pandemic, there was not enough time to repeat the experiment, especially the last part (Big dye sequencing). Further, analysis can defiantly improve the results and can give a food premises micro-organism for spontaneous beer formation.

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