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TESTING DIFFERENT CULTURE MEDIA FOR ISOLATION OF *ZYGOSACCHAROMYCES* SPECIES

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Abbreviations and Acronyms

BSA	Bismuth Sulfite Agar
DFMHS	Department of Food Microbiology, Hygiene and Safety
HCCA	Cyano-4-hydroxy-cinnamic acid
M1	Medium One Agar
M2	Medium Two Agar
M2[a]	Modified M2 agar with Glacial acetic acid
M2[b]	Modified M2 agar without Glacial acetic acid
M3	Medium Three Agar
M4	Medium Four Agar
MALDI -TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
NCAIM	National Collection of Agricultural and Industrial Microorganism
WL Nutrient Agar	Wallerstein Laboratory Nutrient Agar
YEPD	Yeast Extract Peptone Dextrose agar

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1. INTRODUCTION

Zygosaccharomyces species are important yeasts commonly found in various food products, including fermented foods such as soy sauce, miso and sake. They are also known to cause spoilage in high-salts and high-sugar food products, such as pickles and dried fruits (Lianou et al., 2016). Therefore, accurate identification and the isolation of these yeasts are essential for ensuring the quality and safety of food products.

To isolate *Zygosaccharomyces* species from food sample, appropriate and specific culture media need to be developed. These media should support the growth of *Zygosaccharomyces* species while inhibiting the growth of other microorganisms commonly found in the food samples. Moreover, the media should be selective enough to differentiate between different species of *Zygosaccharomyces* (Pfannebecker et al., 2016)

The aim of this study is to compare different culture media for isolation and to get some visible morphological differences amongst of *Zygosaccharomyces* species which requires a thorough understanding of their nutritional requirements and growth characteristics. Different carbon and nitrogen sources, and other nutrients can be added to the media to optimize the growth of *Zygosaccharomyces* species. In addition, pH and temperature conditions should be optimized to ensure maximum growth of the yeasts.

There is a need for new and improved media for selectively isolating various groups, genera, species and /or strains of fungi capable of growing under specific environmental conditions. Selective agents such as antibiotics or fungicides can also be added to the media for inhibiting the growth of other microorganisms. Moreover, specific indicators can be used in the media to differentiate between diverse species of the *Zygosaccharomyces* genus based on their morphological or biochemical characteristics.

2. OBJECTIVE OF THE STUDY

The main goal of the study was to test the applicability of different culture media which can be used for differentiation of spoilage-causing *Zygosaccharomyces* species. By checking specific media formulations that can help to distinguish between different *Zygosaccharomyces* species and/or strains potentially responsible for food spoilage selection of the best medium can be performed.

Different strains have diverse metabolic profiles and preferences, and thus require different nutrients to grow. This can be exploited by designing media formulations that are tailed to the specific metabolic requirements of different strains.

In practice, this can involve testing a range of culture media that differ in their nutrient composition and other factors such as pH, temperature, additives, carbon, and nitrogen sources and comparing the growth of different *Zygosaccharomyces* strains on these agar media. By evaluating the differences of colony morphologies, effective media formulation for differentiating between strains and potentially identifying those strains responsible for spoilage can be identified.

The ability to differentiate between spoilage-causing strains of *Zygosaccharomyces* is very important for food quality control and safety, which can help the producers to target their interventions more effectively, for example adjusting storage conditions and introducing specific antimicrobial agents, thus reducing waste and preventing the spread of foodborne illnesses.

In order to achieve my goals, I planned to take the following steps:

1. Perform the identification of the isolates by MALDI-TOF MS
2. Conduct a comprehensive review of the relevant literature of different culture media and their applicability for differentiating *Zygosaccharomyces* species to be investigated
3. Selection of the culture media based on their composition and potential for supporting growth and differentiation of *Zygosaccharomyces* species
4. Inoculation of the selected culture media with different strains of *Zygosaccharomyces* species
5. Observe and record any observable growth and differentiation of *Zygosaccharomyces* species and/or strains and draw a conclusion based on the results obtained.

3. LITERATURE REVIEW

3.1. Yeasts

Food scientists and biotechnologists have extensively studied yeasts over recent decades. Yeasts are unicellular fungi reproducing asexually by budding or fission, and sexually by spore formation. Emil Christian Hansen's studies, over a span of 30 years, provided insight into the biological features of yeasts and facilitated their differentiation and their characterization as species. Currently the number of known yeast species exceeds 2,000 (Péter, 2022). Most yeast species belong to Ascomycotina (Deák & Péter, 2013; Kurtzman & James, 2006), and a few are basidiomycetes (Fell et al 2000). Some common yeast genera are *Saccharomyces* (used in baking and brewing), *Candida* (associated with infections in humans), and *Cryptococcus* (causes cryptococco humans and animals) (Oren, 2011).

Yeasts are the most important group of microorganisms exploited for commercial purposes including fermentation, biotechnology, and medicine (Walker & Stewart, 2016). They are found in diverse habitats, such as soil, water, plants and animals, and can also be associated with human diseases (Perricone et al., 2017). Yeasts bring about the leavening of bread, and the fermentation of wine and beer that are essential parts of everyday life (Walker & Stewart, 2016). In addition to play a crucial role in the production of fermented foods and beverage, yeasts are also sources of a wide range of valuable products and useful ingredients made by the various branches of biotechnology. Their benefits to human, however, are counterbalanced to some degree by the detrimental role they play in the spoilage of processed and stored foods (Deák, 2007).

Yeasts are eukaryotic unicellular microfungi that are widely distributed in the natural environment (Perricone et al., 2017). Currently, over 2000 yeast species are known, but this represents a fraction of yeast biodiversity on earth (Boekhout et al., 2021; Péter, 2022; Walker, 2009). Although yeasts are primarily unicellular, some may develop hyphae or pseudohyphae. True hyphae are characterized by the lack of a constriction at the cross walls, whereas pseudohyphal cells are formed by budding and elongation, and show a constriction at the attachment of cells.

Yeasts are traditionally characterized, classified, and identified by morphological and physiological criteria. *Zygosaccharomyces* is a member of the Saccharomycetaceae, and most closely related to *Torulaspora*, *Zygotorulaspora*, *Vanderwaltozyma* and *Tetrapisispora*.

Phylogenetic *relationship among* the currently recognized *Zygosaccharomyces* species from analysis of LSU rRNA gene DI/D2 domain. Bar, 1% nucleotide sequence divergence. Evolutionary analysis was conducted in MEGA6 as shown in **Figure 1** (Péter, 2022).

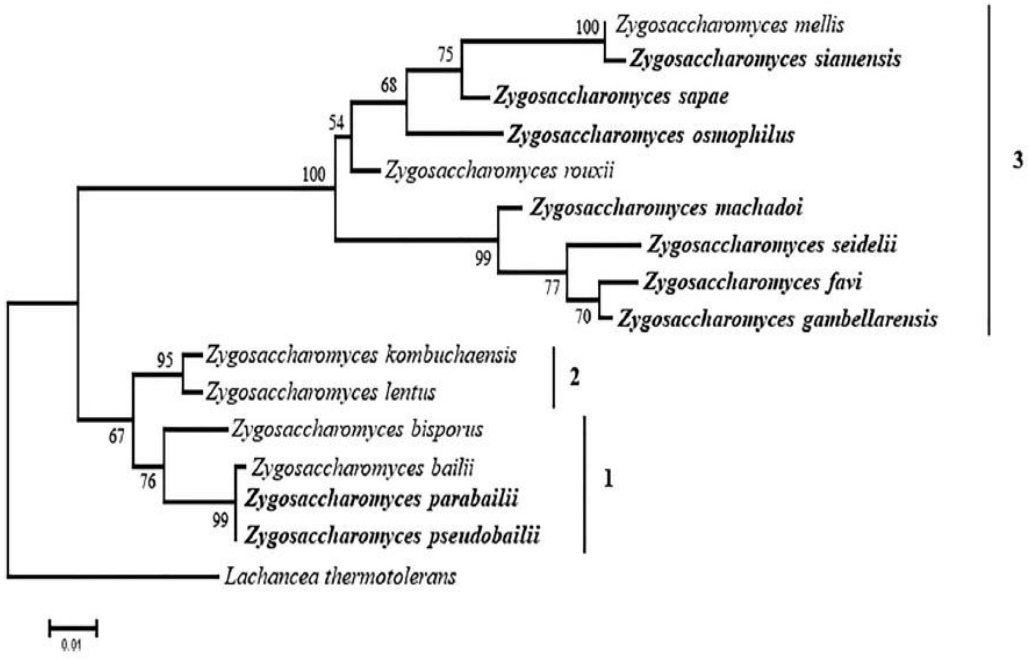


Figure 1. Phylogenetic tree showing relationship among the currently recognized *Zygosaccharomyces* species

Phylogenetic tree showing relationship among the currently recognized *Zygosaccharomyces* species and other species of closely related genera based on D1/D2 LSU rRNA gene sequences. Bar, 1% nucleotide sequence divergence. Evolutionary analysis was conducted in MEGA6 as shown in Figure 2. (Kuanyshev et al., 2017).

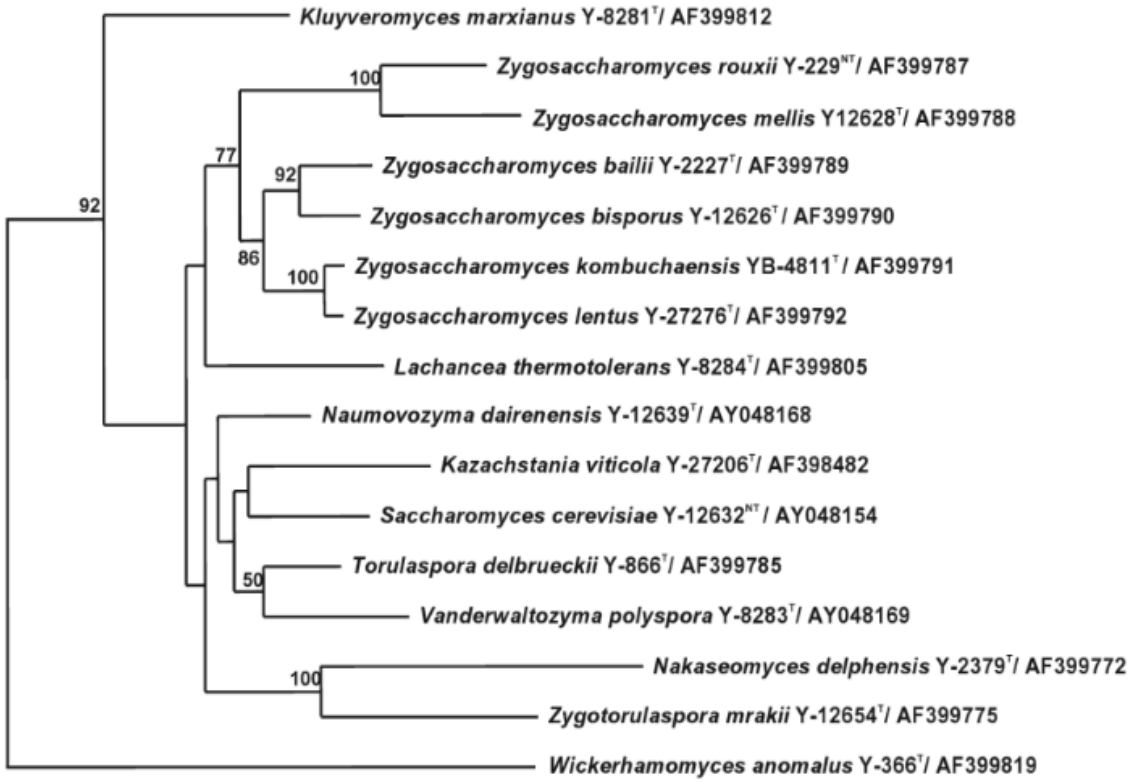


Figure 2. Phylogenetic tree showing relationship among the currently recognized *Zygosaccharomyces* species and other genera

3.2. Classification of spoilage yeasts species

The genus *Zygosaccharomyces* was described by Barker in 1901 (James & Stratford, 2011). The genus shows multilateral budding typical to *Saccharomyces*. Typically, it has 'dumbbell'-shaped asci that arise from ascospores per conjugant. The ascus morphology is not always typical to the genus, and it is sometimes difficult to separate species of *Zygosaccharomyces* from those of other genera (Sá-Correia et al., 2014).

Food and beverage spoilage yeasts often show some degree of specialization. Species of *Zygosaccharomyces* usually colonize high-sugar and high-salt containing products such as fruits and their concentrates, dried fruits, honey, jams and preserves, sugar syrups, salad dressing as well as wine, and are characterized as fermentative spoilage yeasts (Petruzzi et al., 2017).

Yeasts of the genus *Zygosaccharomyces* have a long history of spoilage in the food and beverage industries, including several species that are among the most troublesome food spoilage organisms. Spoilage resulting from growth and metabolic activity of *Zygosaccharomyces* species is widespread (Solieri, 2021), which leads to significant economic losses in the food industry and to a reduction of food supplies worldwide (Escott et al., 2018).

Other spoilage genera include; *Candida*, which contains several species such as *Candida albicans*, *Candida tropicalis*, *Candida krusei* etc. Some *Candida* can cause spoilage in dairy products, juices and baked goods. Others like *Saccharomyces*, which includes species such as *Saccharomyces cerevisiae*, *Saccharomyces uvarum* commonly used in the food industry for fermentation, but can also cause spoilage in wine and fruit juice (Hernández et al., 2018). *Debaryomyces* genus (*Debaryomyces hansenii* and *Debaryomyces fabryi* also known for causing spoilage in cheese and meat products (Patterson, 2005).

The number of species belonging to genus *Zygosaccharomyces* has increased rapidly over the past years, and nowdays genus based on a study made in 2022 includes a total of 15 species*: Z. bailii, Z. bisporus, Z. favi, Z. gambellarensis, Z. kombuchaensis, Z. lentus, Z. machadoi, Z. mellis, Z. osmophilus, Z. parabailii, Z. pseudobailii, Z. rouxii, Z. sapae, Z. seidelii and Z. siamensis.* However, *Z. rouxii* and *Z. bailii* are the most relevant and emerging in the context of the spoilage of food and beverages (Péter, 2022). They also can contribute to beneficial effects during the production of some foods. For example, *Z. rouxii* significantly contributes to the formation of aroma compounds during soy sauce fermentation and converts the sugars available in grapes to

ethanol, the substrate for acetic acid bacteria during production of balsamic vinegar. *Z. rouxii* has also been considered as a starter culture for low-alcohol or alcohol-free beer and baking yeast, while *Z. bailii* plays a positive role during kombucha fermentation by producing ethanol, which is subsequently converted to acetic acid by bacteria (Péter, 2022).

Out of recently isolated species, *Z. pseudobailii* is closely related to *Z. sapae* species. Some of these species affect the food and beverage industries as spoiling microorganisms, and others are associated with fermentation. In this way, from the osmophilic yeasts, being the first cause of fruit juice spoilage, the genus *Zygosaccharomyces* is the most frequently described (Wang, Guo, Yuan, & Yue, 2016).

3.3. Food spoilage activity of *Z. bailii*, *Z. rouxii* and *Z. lentus*

The process of food quality deterioration which leads to changes in nutritional and organoleptic properties is called food spoilage. *Z bailii* is found among the spoilage yeasts that are particularly troublesome in acidic foods and beverages, especially mayonnaise, salad dressings, pickled vegetables, tea, fruit drinks, fruit concentrates and dairy products. In contrast, *Z. rouxii* spoilage is more closely associated with high sugar or salt-based ingredients and finished products like fruit concentrates, syrups, candied fruit pieces and confectionaries such as chocolate (Sá-Correia et al., 2014)

3.4. Characteristics of *Zygosaccharomyces* species

3.4.1. Physiological and morphological characteristics of *Z. bailii*, *Z. rouxii* and *Z. lentus*

Amongst the food and beverage spoiling *Zygosaccharomyces* species, *Z. bailii* possesses the most pronounced and diversified antimicrobial resistance attribute and has the ability to resist antiseptic compounds such as sulphur dioxide, benzoic acid and sorbic acids. Moreover, *Z. bailii* is able to tolerate relatively high ethanol concentrations and high temperatures, and vigorously ferment sugars, thus it is one of the most significant threats in wine industry (Palma et al., 2017; Sá-Correia et al., 2014). Under the microscope, the vegetative cell morphology of Z. *bailii* is usually ellipsoid, non-motile, small in size (3.5-6.5) × (4.5-11.5) µm in Fig. 3 (James & Stratford, 2011). The yeast cells and conjugated asci with ascospores are spherical to ovoid and smooth in appearance which are persistent, after 7 days incubation at 25 ^{o}C on corn meal agar. Cells of the food spoilage yeast

Z. bailii as shown in Fig.3. The bright field image was taken at 100x magnification, using the epifluorescence microscope Zeiss Axiovert 135 TV (DTDT, 2010, Internet 1) as shown in Fig. 3.

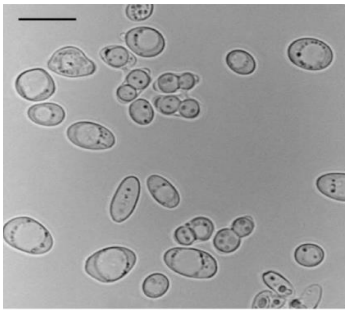


Figure 3. The bright field image of *Z. bailii* taken at 100x magnification

Besides *Z. rouxii* being osmophilic and xerophilic, it is also considered to be acid tolerant. Species can adapt and grow in acidic media at pH value of 2.2 or even as low as 1.8, being the reason why this species could spoil products such as grape juice concentrates (Rojo et al., 2017). Water activity (a_w) tolerance is one of the physiological differences between the species *Z. rouxii* and *Z. bailii*. The species *Z. rouxii* can tolerate low water activity environments, whereas *Z. bailii* requires environment with (a_w) of at least 0.85. This characteristic makes it difficult for *Z. bailii* to survive in high sugar foods such as syrups and candied fruits.

The yeast species *Z. rouxii*, as well as other osmotolerant microorganisms, adjusts its internal osmotic pressure to tolerate high concentration of salts (NaCl) of about 20% (w/v). It is a halotolerant yeast. It can also grow in yeast extract-peptone-dextrose (YPD) medium containing sodium chloride of up to 18% (w/v) (18-YPD). The mechanism is the efflux of sodium cations (Na^+) from cells under high concentration of salt. A change in the fluidity of the lipid-containing cell membranes has been observed when exposing yeast cells to 15% NaCl which is due to a decrease in the degree of saturation and an increase in ergosterol concentration as well as a decrease in the phospholipid to protein ratio. The accumulation of glycerol as a compatible solute (protect the cell against lysis) is the mechanism that *Z. rouxii* follows to survive to high osmolarity (Escott et al., 2018; Stratford et al., 2019).

As already described, the physiological characteristics of *Z. rouxii*, comprising the osmotolerance, the xerophilic ability, the fructophilic capacity and the week-acid tolerance, are responsible for causing food spoilage (Escott et al., 2018). It also tolerates high concentration of sugar and salts;

hence it is a widely occurring spoilage yeast. Colonies of *Z. rouxii* are globose, raised, smooth, and has an entire margin and creamy colour. (Escott et al., 2018).

Another feature of *Z. rouxii* is the production of volatiles in high sugar food matrices at an early stage; this would indicate the presence of spoilage yeast *Z. rouxii*. Ethanol, acetone, ethyl acetate, acetaldehyde, or 3-methyl-1-butanol could be detected by analytical techniques and even through smell of odour. The yeast species *Z. rouxii* is usually haploid and heterothallic. Under the microscope, the vegetative cell morphology of Z. *rouxii* is usually spherical to ovoid, and occur singly, in pairs or in small clusters. The asci ascospores are persistent and often conjugated after 7 days of incubation at 25 ^{o}C on corn meal agar, the conjugation occurs between two separate cells, or between a cell and its bud in Fig. 4. Cell morphology *Zygosaccharomyces rouxii* NCYC 720 can be seen in Fig. 4, where yeast cells and conjugated asci with ascospores after 7 days on corn meal agar at 25 ^{o}C are indicated. Bar is equal with =10 µm (James & Stratford, 2011).

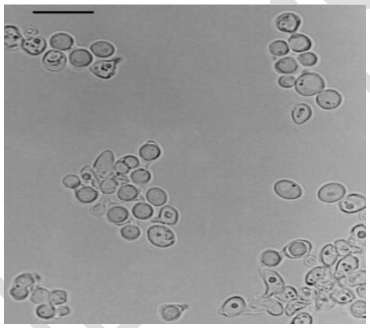


Figure 4. Cell morphology of *Zygosaccharomyces rouxii* NCYC 720, Bar=10 µm

Z. rouxii, similarly to *Z. bailii*, has a high resistance to different chemical compounds used as food preservatives (Stratford et al., 2013). Hydroxycinnamic acids, such as, caffeic acids and p-coumaric acid, have a rather low inhibitory effect around 15%, whilst preservatives like potassium sorbate, sodium benzoate, dimethyldicarbonate and vanillin can inhibit the growth of this yeast species up to 40% (Rojo, et al., 2014). The acetic acid has an impact in its respiratory activity of halo-tolerant yeast *Z. rouxii* R-1, and it also inhibits the formation of cytochromes, hence it is significantly inhibited, and its growth is considerably reduced in the presence of >0.5% acetic acid and media containing NaCl >18% (Kusumegi, Yoshida, & Tomiyama, 1998).

Z. lentus in Figure 5. is closely related to *Z. bailii*, sharing many of its spoilage characteristics, namely osmotolerance, resistance to benzoic and sorbic acids. Unlike *Z. bailii*, *Z. lentus* displays slow growth under aerobic conditions and it is not able to grow at 30 ^{o}C, but it has remarkable ability to grow even if slowly at low temperature (4 ^{o}C). Consequently, it is more important than *Z. bailii* from the viewpoint of spoilage in the case of refrigerated foods. *Z. lentus* has been reported as being unable to grow with 1% (v/v) acetic acid differently from *Z. bailii*, but, at optimum growth temperatures, some strains of *Z. lentus* are even able to surpass *Z. bailii* acetic resistance (Sá-Correia et al., 2014). Under the microscope, cell morphology of Z. *lentus* usually forms a distinctive conjugation tube while other cells contain two or four smooth ascospores. Scanned Electron micrograph of *Z. lentus* IGC 5316, a strain isolated from spoiled wine (James & Stratford, 2003) is shown in Fig. 5.

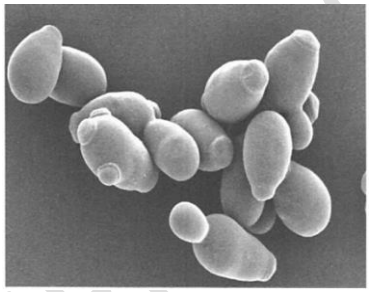


Figure 5. Cell morphology of *Z. lentus* IGC 5316, Bar 10 µm

Photomicrograph of cell morphology of Z. *lentus* NCYC D2627^{T} grown on malt extract agar after 10 days at 22 ^{o}C can be seen on Fig. 6. Many cells have formed distinctive conjugation tubes, while other cells contain two to four smooth ascospores, (Steels et al., 1999).

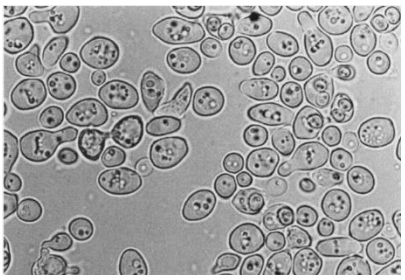


Figure 6. Photomicrograph cell morphology of *Z. lentus* NCYC D2627T, Bar 10 µm

The physiological characteristics between *Z. bailii* and *Z. lentus* are listed in Table 1 (James & Stratford, 2003; Steels et al., 2002).

Table 1. Comparison of the physiological characteristics between of *Z. bailii* and *Z. lentus.*

Z. bailii	***Z. lentus***
Moderate osmotolerance	Osmotolerance, ability to grow in the presence of 60% w/v sugar
Grows fast in aerobic condition	Grows slowly in aerobic condition at temperature above 25 ^{o}C
Does not spoil refrigerated foods	Spoils refrigerated foods (4 ^{o}C), originally established as a new taxon by growth at 4 ^{o}C
Resistant/sensitive to both acetic and sorbic acid	Resistant to both acetic and sorbic acid (Weak acid preservatives)

According to Steels et al 2002, *Z. lentus* possesses typical *Z. bailii* characteristics, displaying extremely poor growth in shaken culture under aerobic conditions and failing to grow in the presence of 1% acetic acids (a diagnostic test used to distinguish *Z. bailii* from other *Zygosaccharomyces* species). To date, nine isolates of *Z. lentus* have been identified and characterized, of which five are known to have originated from spoiled food products (i.e., orange squash, orange juice, tomato ketchup and wine (Steels et al., 1999). However, as mentioned earlier in Table 1, *Z. lentus* will grow a bit slowly at low temperature (i.e., 4 ^{o}C). Such ability therefore raises the possibility that this recently described *Zygosaccharomyces* species could pose a real threat to the food and drink industries as a spoilage agent of chilled products.

One of the most obvious effects observed in food products after the contamination of spoilage yeast is the production of excess gas. This gas compromises the integrity of the food package as it can swell containers, and it could also be responsible for “blown” can or exploding glass bottles. This excess gas is the result of the fermentation of sugars by yeasts during the product’s shelf-life (Escott et al., 2018). The volume of gas produced is variable and so is the pressure inside the food

package; this effect depends on the fermentative yeast species and their fermentation power. In this matter, three *Zygosaccharomyces* species (*Z. lentus*, *Z. bailii*, and *Z. rouxii*) produce larger amounts of gas in comparison to other spoilage yeast genera, as evidence of the high fermenting capacity of *Zygosaccharomyces* genus in food with high sugar content (Escott et al., 2018).

3.4.2. Taxonomic and ecological characteristics of *Zygosaccharomyces* species

The general taxonomic properties of *Zygosaccharomyces* are identical to ubiquitous food yeast genera such as *Saccharomyces*, *Candida*, and *Pichia*. Macro- and micromorphological observations differentiate *Zygosaccharomyces* from other yeasts, or individual species within the genus. On various mycological agars, colonies are smooth, round, convex and cream coloured. Microscopic observation shows large ovoid elongated cells and multilateral budding. All the species of *Zygosaccharomyces* ferment glucose and produce asci with one or four globose or ellipsoidal ascospores inside. *Z. bailii* and *Z. rouxii* can be distinguished by sucrose fermentation properties, presence/absence of growth at 37 ^{0}C and acetic acid resistance (Sá-Correia et al., 2014).

Some of the key taxonomic, biochemical, and physiological tests required to differentiate *Z. bailii* and *Z. rouxii* food spoilage species are listed in Table 2. (Erickson & McKenna, 1999).

Table 2. Key taxonomic, biological and physiological tests required to differentiate *Z. bailii* and *Z. rouxii* food spoilage species

Test	***Z. bailii***	***Z. rouxii***
Glucose fermentation	Positive	Positive (slow)
Sucrose fermentation	Variable (slow)	Variable
Growth at 37 ^{o}C	Variable	Variable
Growth in presence of 1% acetic acid	Positive	Negative
Water activity (a_w) tolerance	0.80-0.85	<0.80

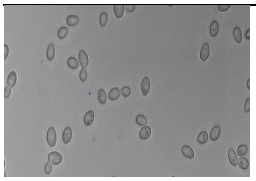
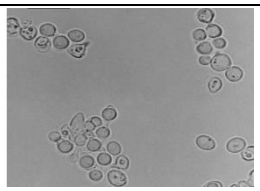
A summary of physiological characterization of spoilage strains of *Z. rouxii* and *Z. bailii* are listed in Table 3. (James & Stratford, 2003; Péter, 2022; Stratford et al., 2019).

Table 3. Physiological characteristics of *Z. bailii* and *Z. rouxii* strains

Z. bailii	***Z. rouxii***
Moderate osmotolerance	Are halotolerant thus exhibit extreme osmotolerance (grow in high salts conc, NaCl about 20%)
High sugar tolerance, *50-60% glucose* (*Fructophilic yeast, prefer fructose to glucose)*	High sugar tolerance, *72-90% glucose*.
Resistance to low pH	Growth in acidic media at pH *2.2 or <1.8*
High tolerance to chemical preservatives, sorbic and benzoic acids (*up to 800-1000mg/L*)	High sorbic acid tolerance, however, quite susceptible to benzoic acid
Grows over a wide range of a_w *0.80-0.85 at 25^oC.*	Are xerotolerant, can grow slowly at a_w 0.62 in fructose & 0.65 in glucose/glycerol *at 25^oC.*
Spoils weak acidic foods such as mayonnaise, salad dressings, pickled vegetables, wine, tea and dairy products	Spoilage is more closely associated with high sugar or salt-based ingredients such as fruit concentrates, syrups, candied fruits and confectioneries such as chocolates.

A summary of colony and cell morphology difference between *Z. bailii* and *Z. rouxii* are listed in the Table 4. (James & Stratford, 2011).

Table 4. Macro- and micromorphological differences of the *Z. bailii* and *Z. rouxii* strains

Z. bailii		***Z. rouxii***	
	Under microscope, the vegetative cells morphology of the yeast strains usually ellipsoid, non-motile.		Under microscope, the vegetative cell morphology of the yeast strains usually circular to elliptical.
	Colony morphology of *Z. bailii* on YEPD agar, at 25ºC after 4-6 days. Usually smooth, round, convex, white to creamy coloured with diameter of 2-3 mm.	Colony morphology of *Z. rouxii* on YEPD agar at 25ºC after 3-4 days. Colonies are creamy white to dull, slightly raised, smooth with wrinkled margin and sub globose to ovoid	
They reproduce asexually by multilateral budding (*Buds can arise from various site on the cells)*		The cells are usually haploid and heterothalic (*Spore formation does not occur in isolated culture)*	
Its cell size varies within a range of (3.5-6.5) x (4.5-11.5) µm and the cells exist singly or in pair, rarely in short chain		Its cell size varies within a range of (2-8) x (2-10) µm and the cells occur singly, in pair or in small clusters	

3.5. Identification of yeasts species using MALDI-TOF MS

Matrix Assisted Laser Desorption Ionization Time of Flight Mass spectrometry (MALDI-TOF MS) is a soft ionization technique that generates a protein-based spectral profile or "fingerprint" that is unique to a given species. Identification of yeast and other micro-organisms is based on acquisition of quality protein spectrum from unknown isolates and comparison of this spectrum to a reference library of defined spectra (Buchan & Ledeboer, 2013). Analysis of yeast or other micro-organisms can often be accomplished by direct application of the isolate to a target plate along with α-cyano-4-hydroxycinnamic acid or other matrix material. The excitation of the matrix material by a laser catalyses charge transfer to the analyte and desorption from the target plate, thus producing charged particles of various sizes that are the basis for creation of spectral profiles. This method has been applied for the identification of a wide variety of bacterial isolates with great success. The comparison of two commercially available methods, the Brucker Biotyper (Bruker Daltonik, Bremen, Germany) and Vitek MS (bioMérieux, Marcy I'Etoile, France) have demonstrated similar performance for the identification of bacterial isolates. Some studies have

found the Bruker Biotyper to provide a higher proportion of “high confidence” identification (94.4% versus 88.8%) for isolates tested and among nonfermenters (97.0% versus 89.5%) (Cherkaoui A, 2010). Regardless of the system used, the identification rate for routine clinical bacterial isolates using MALDI-TOF MS approaches 95% and identification can be completed in less than 10 minutes and with a substantial savings in cost per identification. The MALDI Biotyper system can provide an accurate method for the identification of clinical pathogenic isolates (Li et al., 2019).

3.6. Culture media for isolation of yeasts

Yeasts isolation culture media are used for selectively grow and isolate different type of yeasts from a variety of sources including food, beverage and environmental samples. Previous studies have been conducted on different types of culture media such as Potato Dextrose Agar (PDA), containing potato infusion and dextrose as the main nutrient along with agar as a solidifying agent, it has a neutral pH which allow growth of wide range of microorganism including, yeast. It is used widely for isolation and cultivation of yeasts and fungi (Brinda et al., 2021).

Other culture media used for cultivation and isolation of different types of yeasts include: Yeast Malt Agar (YMA), (containing malt extract, yeast extract and peptone as source of nutrients and agar as solidifying agent, (Petkova et al., 2022) and, Rose Bengal Chloramphenicol (RBC) Agar (Peters et al., 2019). However, no one medium is satisfactory for detection or enumeration of all yeasts and moulds in all foods. Antibiotic-supplemented media are superior to acidified media for enumeration of yeast and moulds. Dichloran 18% glycerol agar performs well for enumerating moderately xerophilic yeasts. Fastidious xerophiles require media containing high concentration of sugars and /or sodium chloride. These media have been formulated to detect potential yeast strains but increased selectivity and specificity of them for detection these strains are needed. The spread plate technique is strongly preferred over the pour plate technique for enumerating yeasts.

The recommended incubation temperature is 25 ^{o}C but incubation time between plating and counting colonies ranges from 5 days to 4 weeks (Beuchat, L. R, 1992). Relatively few media are available specifically for enumerating or at least facilitating the growth of yeasts at the expense of moulds and bacteria. However, since yeasts may be predominant microorganisms in some foods, such as fruit juice concentrates, special media for enumeration of yeasts are desirable. Davenport (1980) outlined a guide to media and methods for studying yeasts in foods. Oxytetracycline

glucose yeast extract (OGYE) agar (Mossel et al., 1970; Baird et al., 1987) has been widely used for many years, especially in Europe, as a general medium for enumerating yeasts. The medium loses its bacteriostatic effect if incubated at temperatures greater than 25 ^{o}C. Moulds will also develop colonies on OGYE agar. Tryptone glucose yeast extract (TGY) agar supplemented with antibiotics can also be used successfully for products containing high populations of yeasts.

Acid-resistant yeasts, particularly *Zygosaccharomyces bailii* and to a lesser extent *Z. rouxii*, cause spoilage of foods containing benzoic and sorbic acids. These yeasts can be enumerated with some success on malt extract agar supplemented with 0.5% acetic acid as noted by Samson et al. (1992). Other media used to enumerate acid-resistant yeast are acidified (0.5% acetic acid) TGY agar (D eak and Beachat, 1996; Hocking, 1996) and *Zygosaccharomyces bailii* agar (Erick-son, 1993). Modification of TGY agar has enhanced its performance (Makdesi and Beuchat, 1996a; 1996b; 1996c); however, a highly selective medium for enumerating acid-resistant yeast has yet to be developed(Hernandez & Beuchat, 1995; Zuehlke et al., 2013).

In summary, development and testing of culture media for isolation of *Zygosaccharomyce*s species should be optimized for maximum growth and selectivity of the species to ensure their efficient isolation and identification from food samples. Moreover, the culture media should be able to suppress bacterial growth completely without affecting growth of food fungi. It should be nutritionally adequate and support the growth of relatively fastidious fungi(Beuchat, 2003).

4. MATERIALS AND METHODS

4.1. Yeast strains

Yeast strains examined in this study are listed in Table 5. A total of 9 strains were used: 3 strains, belonging to *Z. rouxii* and 5 strains belonging to *Z. bailii*, and one strain belonging to *Z. lentus*. These strains were collected from Department of Food Microbiology, Hygiene and Safety of MATE and the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary).

Table 5. Spoilage yeast strains used in this study and their origin.

Yeast strains[a]	Reference number[b] /[T]	Origin, Country
Zygosaccharomyces lentus	Y120	Department of FMHS, Hungary
Zygosaccharomyces bailii	NCAIM Y.00954^T	NCAIM, Hungary
Zygosaccharomyces bailii	IV/3/D	Department of FMHS, Hungary
Zygosaccharomyces bailii	PM 167	Department of FMHS, Hungary
Zygosaccharomyces bailii	1/3/3	Department of FMHS, Hungary
Zygosaccharomyces bailii	B 216	Department of FMHS, Hungary
Zygosaccharomyces rouxii	NCAIM Y.00729^T	NCAIM, Hungary
Zygosaccharomyces rouxii	AG	Department of FMHS, Hungary
**Zygosaccharomyces rouxii*	IV/3/7	Department of FMHS, Hungary

Abbreviations: NCAIM, National Collection of Agricultural & Industrial Microorganisms, Hungary.

[a] Species identification was confirmed by Matrix assisted laser desorption ionization time of flight mass spectrometry, MALDI-TOF MS (*Bruker MALDI Biotyper MSP Identification Standard Method 1.1*).

[b] Yeasts were isolated from wine origin except NCAIM strains (Y.00954^T & Y.00729^T).

[T] Type of strains of species (collected from NCAIM)

4.2. Culture medium for maintenance of yeast strains

Yeasts were maintained at 4 ^{o}C for subculturing. Yeast Extract Peptone Dextrose (YEPD) agar slants were prepared containing yeast extract (0.5%, w/v), peptone (0.5%, w/v), glucose (1%, w/v) and agar (1.5%, w/v), and sterilized (121 ^{o}C for 15 min). Cultures were inoculated onto the YEPD agar slants and incubated at 25 ^{o}C for 24 h.

4.3. Culture media used for differentiating *Z. bailii*, *Z. rouxii* and *Z. lentus*

The composition of the media used to get the overview of the similarities and differences of the *Zygosaccharomyces* species is listed in **Table 6** (WL Agar, YEPD, and M1 Agar); **Table 7** (M2 agar and its modified versions); **Table 8** (BSA); **Table 9** (M3 and its modified version, M4 Agar)

Table 6. Culture media composition.

WL Nutrient Agar	**YEPD Agar**	**M1 Agar**
Agar, 10g/L	Agar, (1.5% w/v)	Agar, (1.5%w/v)
Yeast extract, 2g/L	Yeast extract (0.5% w/v)	Yeast Extract, (0.5%w/v)
Glucose, 25g/L	Glucose, (1% w/v)	Fructose, (5%w/v)
Bromocresol green, 0.011g/L	Peptone (0.5% w/v)	Glucose, (45% w/v)
Manganese sulphate, 0.0625g/L		
Magnesium sulphate, 0.0625g/L		
Calcium chloride, 0.0625g/L		
Potassium chloride, (KCL) 0.275g/L		
Potassium Hydrogen phosphate, 0.55g/L		
Pancreatic digest of casein, 2.5g/L		

Abbreviation: WL-Wallerstein Laboratory, Final pH 5.5±0.2 (25^{o}C), BBL Microbiology Systems, Difco Laboratory and Oxoid Unipath. **YEPD**- Yeast Extract-Peptone-Dextrose (D-glucose) **M1**-Media one referred as Z. rouxii agar by (Beuchat 1992).

Table 7. Culture media composition.

M2 agar	Modified M2	Modified M2[a]	Modified M2[b]
Agar, 1.5%w/v	Agar, 3.0%w/v	Agar, 1.5%w/v	Agar, 1.5%w/v
Yeast Extract, 0.5%w/v	Yeast Extract, 0.5%w/v	Yeast Extract, 0.5%w/v	Yeast Extract, 0.5%w/v
Glucose, 10% w/v	Glucose, 10% w/v	Glucose, 10% w/v	Glucose, 10% w/v
Potassium chloride, 10% w/v	Potassium chloride, 10% w/v	Potassium chloride, 10% w/v	Potassium chloride, 10% w/v
Glycerine, 10%w/v	Glycerine, 10%w/v	Glycerine, 10%w/v	Glycerine, 10%w/v
		Glacial acetic acid, 0.5% w/v	Bromophenol blue indicator, 0.002%w/v
		Bromophenol blue indicator, 0.002%w/v	

M2-Media two as described by (Pfannebecker et al., 2016).

Table 8. Bismuth Sulfite Agar composition.

Ingredients	***Grams per liter***
Beef extract	5.0g/L
Peptone from meat	10.0g/L
D (+) Glucose	5.0g/L
Di Sodium Hydrogen Phosphate	4.0g/L
Iron (II) Sulphate	0.3g/L
Brilliant green	0.025g/L
Bismuth Sulfite Indicator	8.0g/L
Agar	20.0g/L

Abbreviation: Bismuth Sulfite Agar (BSA) pH 7.6±0.2 at 25^{o}C -modification of Wilson and Blair formula.

Table 9. Culture media composition.

M3 Agar	Modified M3	M4 Agar
Peptone, (0.1% w/v)	Peptone, (0.1% w/v)	Peptone, (0.5%% w/v)
Glucose, (30% w/v)	Glucose, (30% w/v)	Yeast extract, (0.25%w/v)
Tween 80, (0.05% w/v)	Tween 80, (0.05% w/v)	Glucose, (10% w/v)
Glycerol, (18% w/v)	Glycerol, (18% w/v)	Agar, (1.5% w/v)
Agar, (1.5%)	Agar, (3.0% w/v)	

Abbreviation: **M3**- Media three as described by (Hernandez & Beuchat, 1995)

M4- Media four as described by (Andrews et al., 1997; Beuchat et al., 1998)

M2^{a}- Modified M2 with Glacial acetic acid. M2^{b}-Modified M2 without Glacial acetic acid

4.4 Identification of the *Zygosaccharomyces* species by MALDI-TOF MS

MALDI-TOF MS (Brucker's Flex series) was used for rapid screening to comprehensively target characterize the strains. Overnight cultures of the different *Zygosaccharomyces* strains were smeared as a thin film directly onto a cleaned MALDI target. The smears were performed in such a way that homogenous spots were visible on the target. Each smear was then overlayed with 1 µL of 70% aqueous formic acid and allowed to dry completely at room temperature. 1 µL of Cyano-4-hydroxy-cinnamic acid (HCCA) matrix solution was applied to each spot, left to dry and after drying the MALDI target was ready and inserted immediately into the equipment and analysed.

Furthermore, the reproducibility of the method was tested by measuring each spot two times. A total of 14 spectra were acquired and compared with the reference database. Spectra were acquired using a Microflex LT mass spectrometer (Brucker Daltonics, Bremen, Germany) according to the standard setting recommended by the manufacturer (method 'MBT-AutoX). The obtained spectra were validated using the Flex Analysis Software to check the calibration constant, visual inspection of outliers or anomalies, and peak shifts.

4.5. Micro and macro morphological characterization of the yeasts.

4.5.1. Inoculation onto YEPD and WL Nutrient media

Small quantities of the overnight cultures of the 9 strains were streaked with sterile loop on the already prepared WL and YEPD agar plates, then incubated at 25 ^{o}C for 24 h, 48 h & 6 days. An Olympus BX41 Fluorescence microscope was used for micro morphology examination of strains at different time intervals (24 h & 48 h,), then further incubated till the 6th day. An Olympus SZX7 Stereo microscope with DP10 camera was used for macro morphology examination of the strains after 24 h, 48 h & 6 days of incubation.

4.6. Preparation of the tested media for differentiation of *Zygosaccharomyces* species

4.6.1. Testing M1, M2, and M4 culture media.

Small quantities of overnight cultures from the 9 strains were streaked with sterile loop on the already prepared agar media (M1, M2, and M4), then incubated at 25 ^{o}C for 24 h, 48 h & 6 days. An Olympus SZX7 Stereo microscope with DP10 camera was used for macro morphology examination of the strains at different time intervals (24 h, 48 h, and 6 days).

4.6.2. Testing modified culture media (M2, M3, M2^{a}, M2^{b}) and Bismuth Sulfite Agar.

The experiment was done using yeast suspension with initial optical density (OD=0.5) as a stock solution. Small quantities of overnight culture from the 9 strains were suspended in sterile water with a sterile loop, vortexed and using optical densimeter to make OD of 0.5. Two-fold dilutions were made from the already prepared solution by pipetting 0.5 ml onto 4.5 ml of sterile water. 10 µl of each dilution were inoculated on the already prepared culture medium, then incubated at 25^{o}C for 24 h, 48 h, & 6 days. An Olympus SZX7 Stereo microscope with DP10 camera was used for macro morphology examination of the strains at different time intervals (24 h, 48 h, and 6 days) in the cases of modified M2 and M3 culture medium, and after 7 days in the cases of M2^{a} and M2^{b} culture media, and Bismuth Sulfite Agar (BSA).

5. RESULTS AND DISCUSSION

5.1 Identification of the *Zygosaccharomyces* species by MALDI-TOF MS

The aims of MALDI analyses were to identify and characterize the *Zygosaccharomyces* species and differentiate them from the other species and/or strains can be found in the reference database. Result of identification for seven *Zygosaccharomyces* strains (from which NCAIM strains were excluded) using MALDI Biotyper are shown in Fig. 7

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
B11 (-) (C)	Z bailii IV 3D (Standard)	No Organism Identification Possible	1.42	No Organism Identification Possible	1.19
B12 (-) (C)	Z bailii IV 3D (Standard)	no peaks found	0.00	no peaks found	0.00
C1 (-) (B)	Z bailii I 3 3 (Standard)	no peaks found	0.00	no peaks found	0.00
C2 (+) (B)	Z bailii I 3 3 (Standard)	Zygosaccharomyces bailii	1.86	No Organism Identification Possible	1.65
C3 (-) (C)	Z rouxii A9 (Standard)	no peaks found	0.00	no peaks found	0.00
C4 (-) (C)	Z rouxii A9 (Standard)	No Organism Identification Possible	1.30	No Organism Identification Possible	1.30
C5 (-) (B)	Z bailii BJ16 (Standard)	no peaks found	0.00	no peaks found	0.00
C6 (+) (B)	Z bailii BJ16 (Standard)	Zygosaccharomyces bailii	1.87	No Organism Identification Possible	1.60
C7 (-) (C)	Z lentus (Standard)	No Organism Identification Possible	1.32	No Organism Identification Possible	1.32
C8 (-) (C)	Z lentus (Standard)	No Organism Identification Possible	1.41	No Organism Identification Possible	1.35
C9 (-) (C)	Z rouxii IV 3 7 (Standard)	no peaks found	0.00	no peaks found	0.00
C10 (-) (C)	Z rouxii IV 3 7 (Standard)	no peaks found	0.00	no peaks found	0.00
C11 (-) (C)	Z bailii PM167 (Standard)	no peaks found	0.00	no peaks found	0.00
C12 (-) (C)	Z bailii PM167 (Standard)	no peaks found	0.00	no peaks found	0.00

Result overview table--continued on next page

Figure 7. Results of *Zygosaccharomyces* strains' identification in two parallels by MALDI-TOF MS

*Yellow means a match with low confidence identification of a Z-score between 1.70-1.99.

* Red means no organism identification possible of a Z-score between 0.00-1.69.

Results for *Zygosaccharomyces* identification showed a match with low confidence identification of a Z score of 1.86 and 1.87 for *Z. bailii* strains, while in the cases of the other strains there were

either no peak found or no organism identification was possible with a Z-score between 0.00-1.65. Most of the strains were not identified based on the database. The database used for identification was developed for clinical application, the software available at the Department of Food Microbiology, Hygiene and Safety was not appropriate for my purposes. Thus, for identification of *Zygosaccharomyces* yeasts MALDI proved to be an insufficient method.

5.2. Macro- & micro morphological characteristics of *Z. bailii* , *Z. rouxii, Z. lentus* strains on WL Nutrient Agar.

5.2.1. Micromorphology of the tested *Zygosaccharomyces* species

Result of micro morphological investigations of the *Z. bailii*, *Z. rouxii and Z. lentus* strains tested on WL Nutrient agar at 25 ^{o}C for 24 h and 48 h are represented in Fig. 8-10 (using Olympus BX41 Fluorescence microscope).

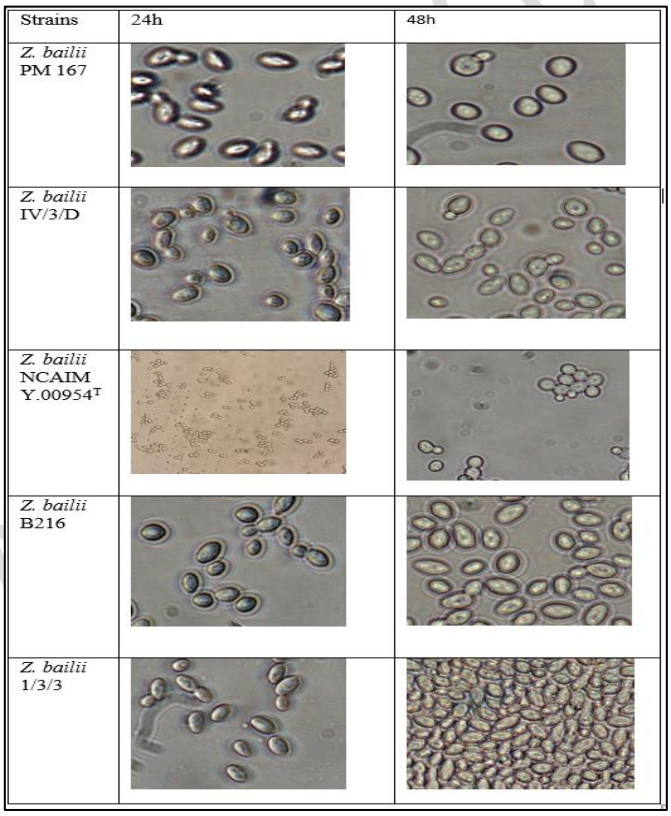


Figure 8. Bright field image of *Z. bailii* strains at 600X magnification

Cells of *Z. bailii* strains (**Figure 8**) had appearance of particulates when grown on WL Nutrient medium, usually as a result of pseudohyphal formation (Gizaw, 2022; James & Stratford, 2011). The size of *Z. bailii* cells were typically small, and it is consistent the observation of (James & Stratford, 2003) who measured a size range of (3.5-6.5) × (4.5-11.5) µm, while the shapes were oval to elongate. The cell arrangement of the strains was either single cells or in pairs with short chains (*Z. bailii* NCAIM Y.00954^{T}). There were clear and visible cells after 24 h except for *Z. bailii* NCAIM Y.00954^{T}.

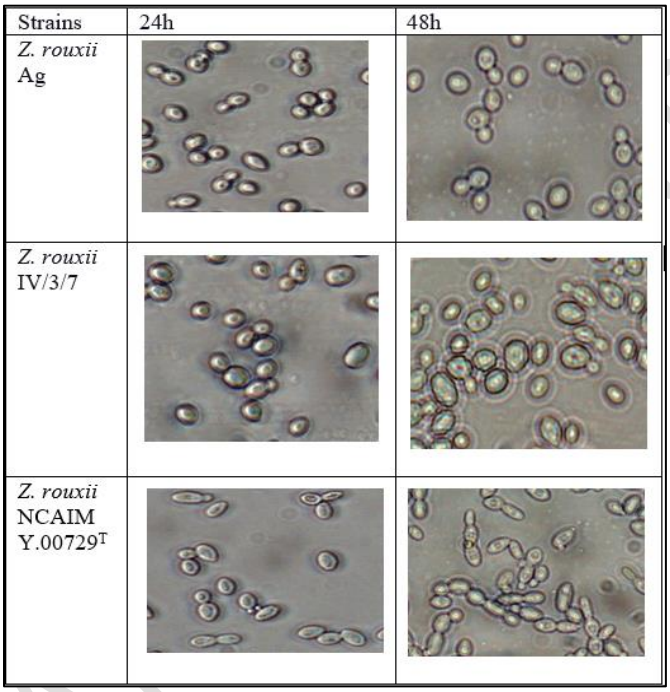


Figure 9. Bright field image of *Z. rouxii* strains at 600X magnification

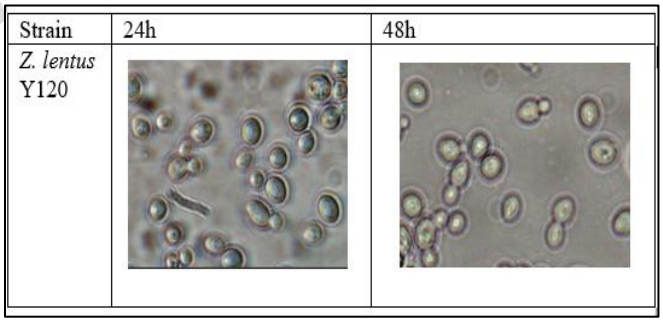


Figure 10. Bright field image of *Z. lentus* Y120 at 600X magnification

Cells of *Z. rouxii* strains **(Figure 9.)** were oval or ellipsoidal, and some were arranged single, in pairs, or in small clusters. *Z. rouxii* Ag *and Z. rouxii* IV/3/7 were oval, unlike *Z. rouxii* NCAIM Y.00729^{T} which were ellipsoidal. There was an observation of conjugated asci with ascospores in between two separate cells or a cell and its bud in **Figure 9**. A previous study reported that asci are persistent and some strains represent haploid and heterothallic (Escott et al., 2018; García-Garibay et al., 2014; James & Stratford, 2011).

Cells of *Z. lentus* Y120 in **Figure 10** were ellipsoidal and was arranged either single or chains of cluster cells. In previous studies it was shown that *Z. lentus* is closely related to *Z. bailii and Z. bisporus* (Sá-Correia et al., 2014).

5.2.2. Macro morphology of *Zygosaccharomyces* species

Results of colony morphology of *Z. bailii, Z. rouxii,* and Z. *lentus* strains on WL agar at 25^{o}C after 24 h, 48 h and 6 days scanned by Olympus SZX7 Stereo microscope and DP10 camera are shown in Fig. 11-13

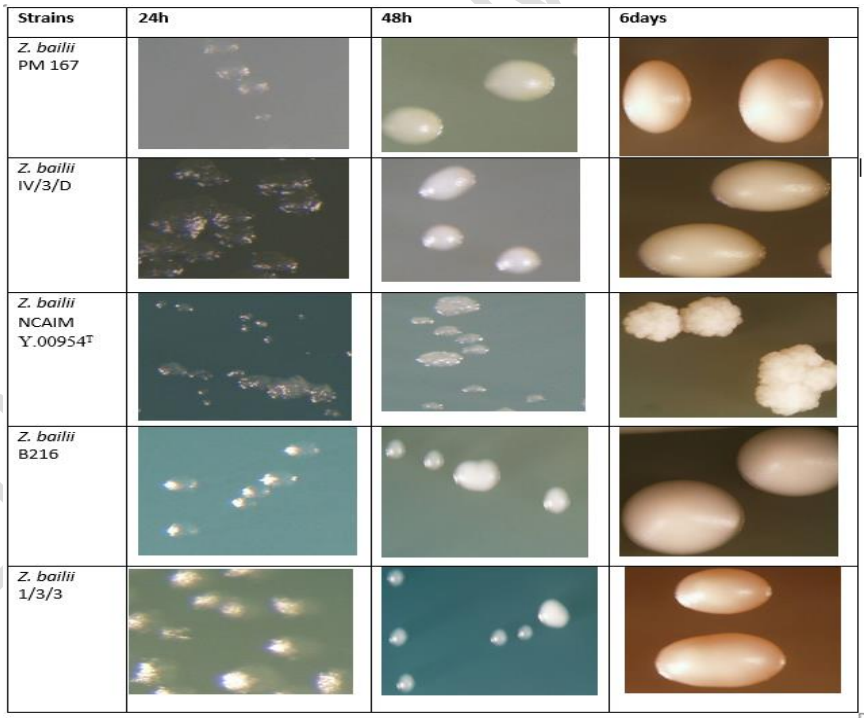


Figure 11. Images of colony morphologies of *Z. bailii* strains on WL Nutrient agar

There was observable and significant difference between *Z. bailii* NCAIM Y.00954^{T} and other *Z. bailii* strains on WL Nutrient agar at 25^{o}C after 48 h and 6 days. *Z. bailii* NCAIM Y.00954^{T} colonies were creamy white in colour, irregular shape, and erose margin with moderate to small size of a diameter of 1-2 mm as shown in **Figure 11.**

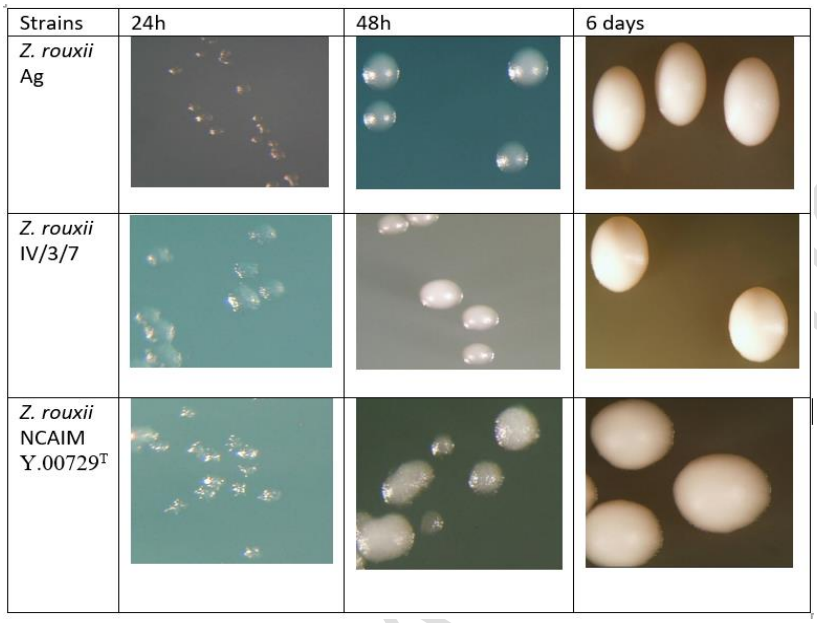


Figure 12. Images of colony morphologies of *Z. rouxii* strains on WL Nutrient agar

Figure 12 shows no significant difference in morphological features of the *Z. rouxii* strains on WL Nutrient agar after 6 days of incubation. However, the viability of the colonies increased with time indicating exponential growth of each strain.

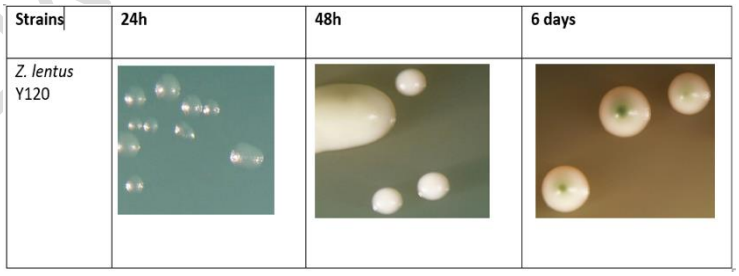


Figure 13. Images of colony morphologies of *Z. lentus* Y120 on WL Nutrient agar

There was a distinct feature of *Z. lentus* Y120 on WL Nutrient agar at 25^0C after 6 days of incubation. *Z. lentus* Y120 colonies had green color at the center (**Fig. 13**). I hypothesized that WL Nutrient agar with 0.011g/L bromocresol green indicator (that turns from green to yellow as the pH decreases, M. J. Kim et al., 2012; O'brien et al., 2023) might have contributed to the green color at the center of the colonies due to the metabolism of the strain. *Z. lentus* is known for its ability to produce organic acids such acetic acids which lower the pH of the medium during its growth.

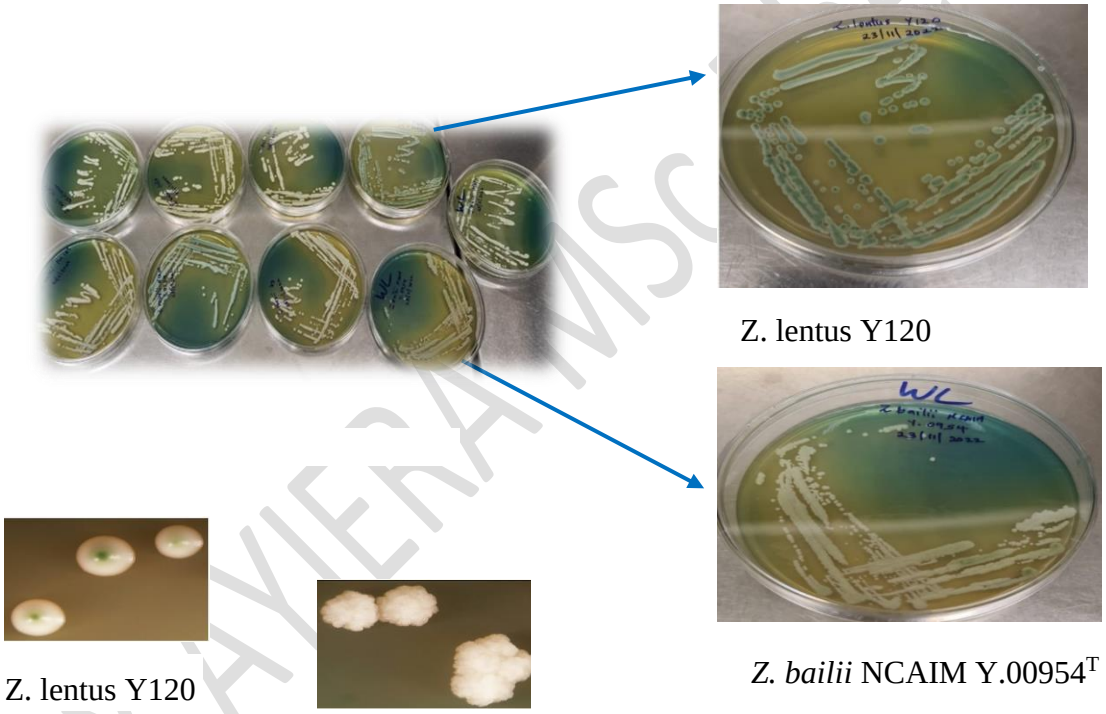


Figure 14. Summary results of Z. *lentus* Y120 and *Z. bailii* NCAIM Y.00954^T on WL agar plates after 6days of incubation

It was noted that WL Nutrient agar after 6 days of incubation, could differentiate the two *Zygosaccharomyces* strains (*Z. lentus* Y120 and *Z. bailii* NCAIM Y.00954^T) from the rest of the investigated ones (Fig. 14).

5.3. Macro morphological difference of *Zygosaccharomyces* species on M1, M2 and M4 media

5.3.1. Colony morphology of *Zygosaccharomyces* species on M1 agar at 25 ^{o}C

Results of colony morphologies of *Z. bailii*, *Z. rouxii*, and *Z. lentus* strains on M1 agar at 25 ^{o}C after 24 h, 48 h and 6 days of incubation scanned by Olympus SZX7 Stereo microscope and documented by DP10 camera are shown in Fig. 15.

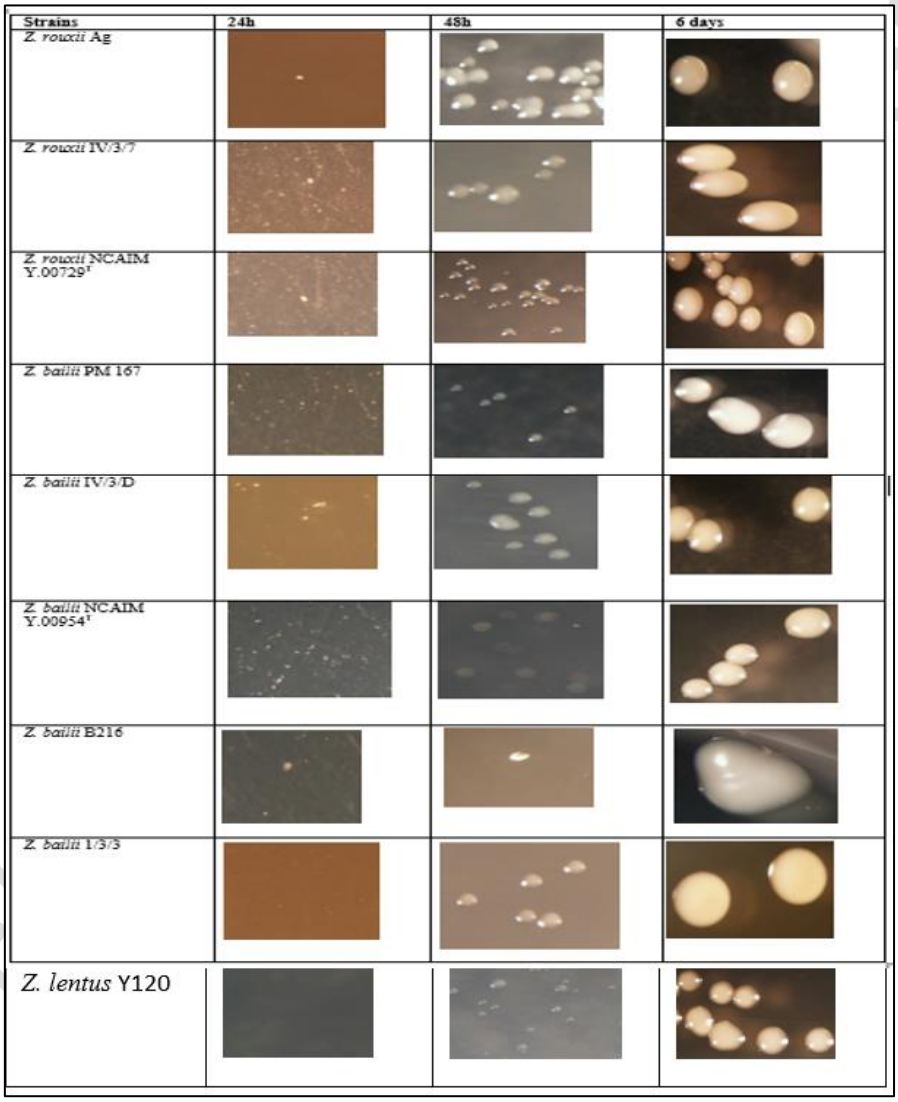


Figure 15. Images of colony morphologies of *Z. bailii*, *Z. rouxii* & *Z. lentus* strains on M1 agar

The results in Figure 15. showed that there were no observable macromorphological differences of *Zygosaccharomyces* strains. However, there was a slow growth rate for *Z. bailii* as compared to *Z. rouxii* strains shown in Figure 15. after 48 h of incubation. Clear colonies with white/creamy colour and moderate to small in size with no unique feature could be observed after 6 days of incubation. *Z. lentus* Y120 also showed slow growth rate with clear colonies after 6 days of incubation.

5.3.2. Colony morphology of *Zygosaccharomyces* species on M2 agar at 25 ^{o}C

Results of colony morphological studies of *Zygosaccharomyces* species on M2 agar at 25 ^{o}C after 6 days of incubation scanned by Olympus SZX7 Stereo microscope and documented by DP10 camera are shown in Fig. 16.

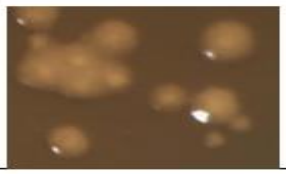
Strains	**6 days**
Z. rouxii Ag	
Z. rouxii NCAIM Y.00729^{T}	
Z. bailii PM 167	
Z. bailii B216	
Z. bailii 1/3/3	
Z. rouxii IV/3/7	-ve
Z. bailii NCAIM Y.00954^{T}	-ve
Z. bailii IV/3/D	-ve
Z. lentus Y120	-ve

Figure 16 Images of colony morphologies of *Zygosaccharomyces* species on M2 agar

Result in **Figure 16.** showed that there was no growth of *Zygosaccharomyces* strains on M2 containing 1.5% w/v agar after 24 h & 48 h and even after 6 days of incubation, some strains could grow while others did not grow and in the cases of those strains that were able to grow, some colonies were not clear, especially *Z. bailii* 1/3/3. I hypothesized that the streaking method was not appropriate for the culture medium. In addition, this medium was very soft and in my opinion the cells may not have been able to anchor themselves properly due to low viscosity resulting to being washed away from their point of attachment, and thus reducing their viability.

In addition, the liquid medium may have led to loss of the yeast cells from the system due to osmotic pressure of the environment which reduced the overall yield (Aggio et al., 2012; Vega et al., 2003). In addition, some strains of *Z. rouxii, Z. bailii and Z. lentus* Y120 also showed negative response to the culture medium. Overal, a very soft growth medium may have negative effects on the yeast growth and survival, potentially leading to reduced biomass production and impaired metabolic activity. Previous studies also reported the negative effect of soft medium on the formation of biofilm which are important for survival and growth of microorganisms, weak biofilms lead make the cells vulnerable to environmental stresses such as temperature, pH or nutrient availability (Kim et al., 2016).

5.3.3. Colony morphology of *Zygosaccharomyces* species on M4 agar at 25 ^{o}C

Results of colony morphology of *Zygosaccharomyces* strains on M4 agar at 25 ^{o}C after 24 h, 48 h, and 6 days of incubation scanned by Olympus SZX7 Stereo microscope DP10 camera are shown in Fig. 17-20.

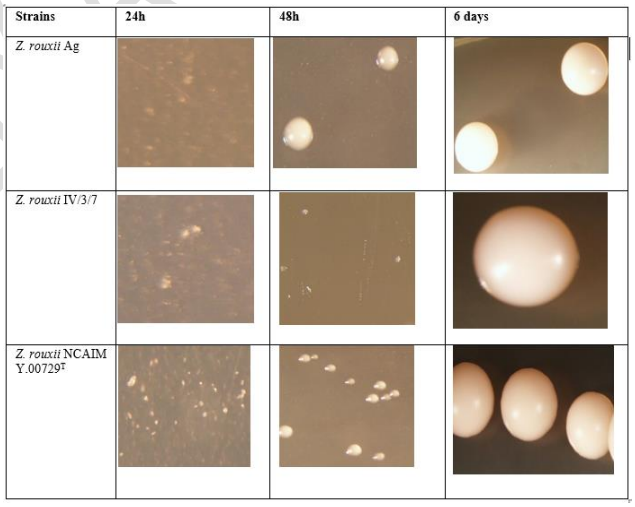


Figure 17. Image of colony morphology of *Zygosaccharomyces rouxii* strains on M4 agar

The results in Figure 17. show a slow growth rate after 24 h of incubation with no clear colonies of *Z. rouxii*. According to Table 7, M4 agar has 10% glucose content, while other tested culture media contained more glucose. However, after 6 days of incubation the colonies of *Z. rouxii* strains appeared on the agar as creamy white to beige colonies, slightly raised, smooth, glistening surface, and mucoid texture with no clear distinction between the strains, despite the size variation of the strains after 48hr of incubation.

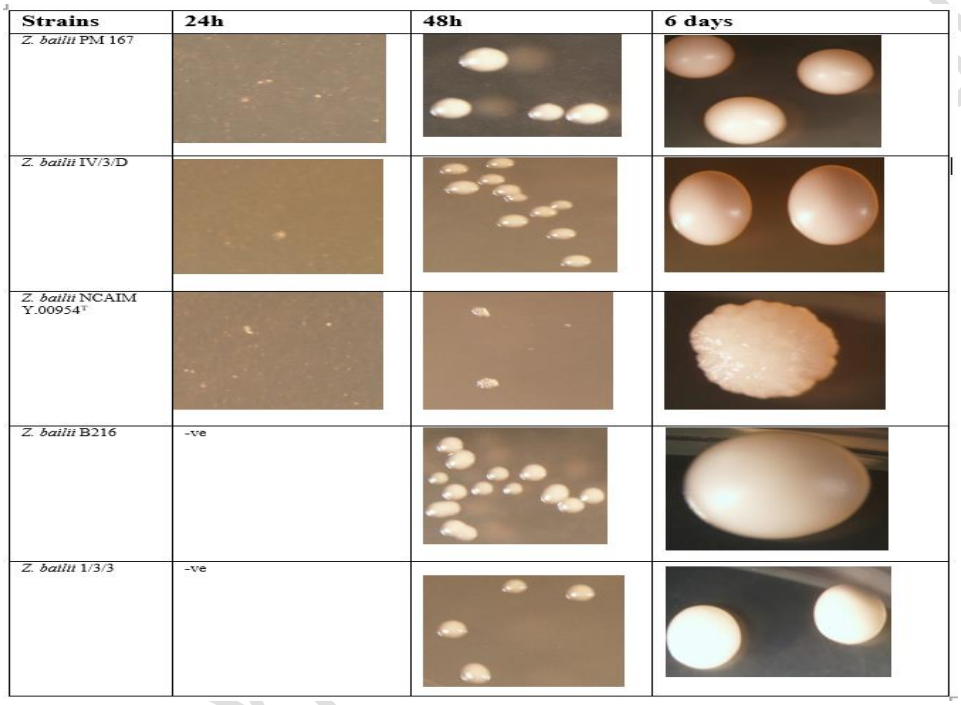


Figure 18. Images of colony morphology of *Zygosaccharomyces bailii* strains on M4 agar

strains	24h	48	6 days
Z. lentus Y120	Negative		

Figure 19. Images of colony morphology of *Zygosaccharomyces lentus* Y120 on M4 agar

The results in **Figure 18.** show that *Z. bailii* strains on M4 agar have similar morphological characteristics, except for *Z. bailii* NCAIM Y.00954^{T}, that has creamy white, mucoid texture and irregular shape with undulated margin. The same strain variation was seen on WL Nutrient agar in Figure **11** and **14**, (in section 5.2.2.).

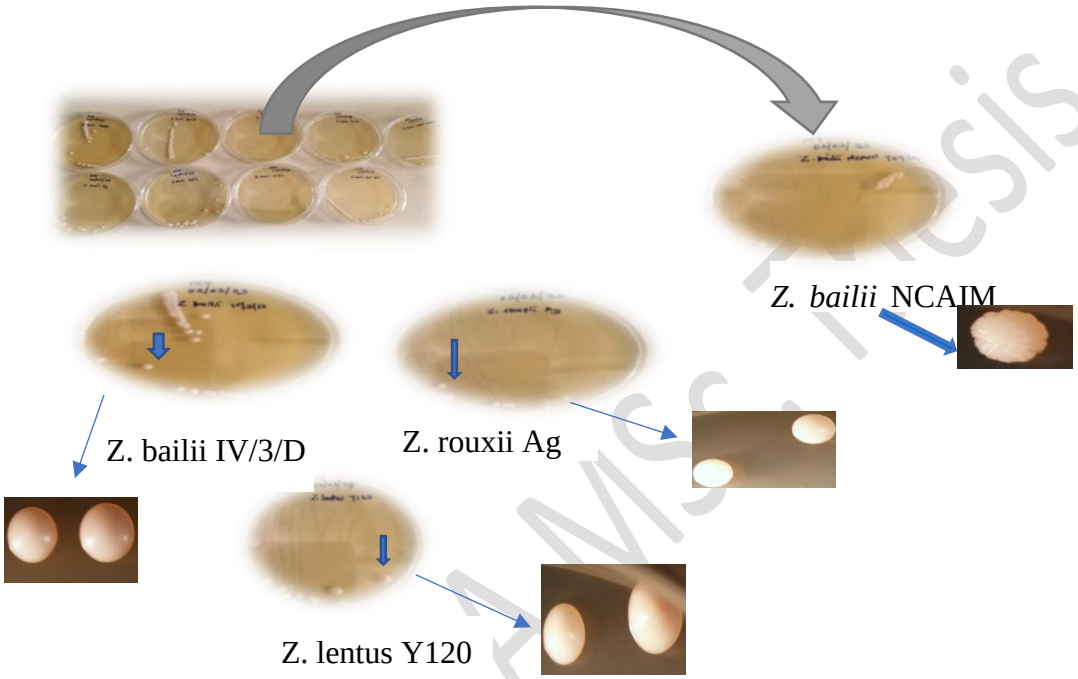


Figure 20. M4 agar plates with *Zygosaccharomyces* species after 6 days of inoculation

Results in Figure 20. show a clear distinct morphological difference between *Z. bailii* NCAIM Y.00954^{T} and other *Z. bailii* strains on M4 agar which is observable on the petri dish without using stereo microscope. There was a unique feature of *Z. lentus* Y120 forming white creamy, umbonate colonies, and as it was observed it turned green at the center of the colony on WL Nutrient agar in Figure 13 & 14, (in section 5.2.2). In addition, Z. *bailii* IV/3/D could be seen as appearing to be buff in color and dull as shown in Figure 20. However, I could not confirm the differences based on colour as colour sometimes is influenced by the environment and the medium composition as it was stated by Steels and coworkers (2002) as well.

5.4. Macro morphological differences of *Zygosaccharomyces* species on modified M2, M3, M2[a], M2[b] and Bismuth Sulfite Agar (BSA).

5.4.1. Colony morphology of *Zygosaccharomyces* species on modified M2 agar at 25 °C

Results of colony morphologi*cal* studies of *Zygosaccharomyces* strains on modified M2 agar containing 3.0% w/v of agar at 25 °C after 24 h, 48 h, and 6 days of incubation scanned by Olympus SZX7 Stereo microscope and documented by DP10 camera are shown in Fig. 21.

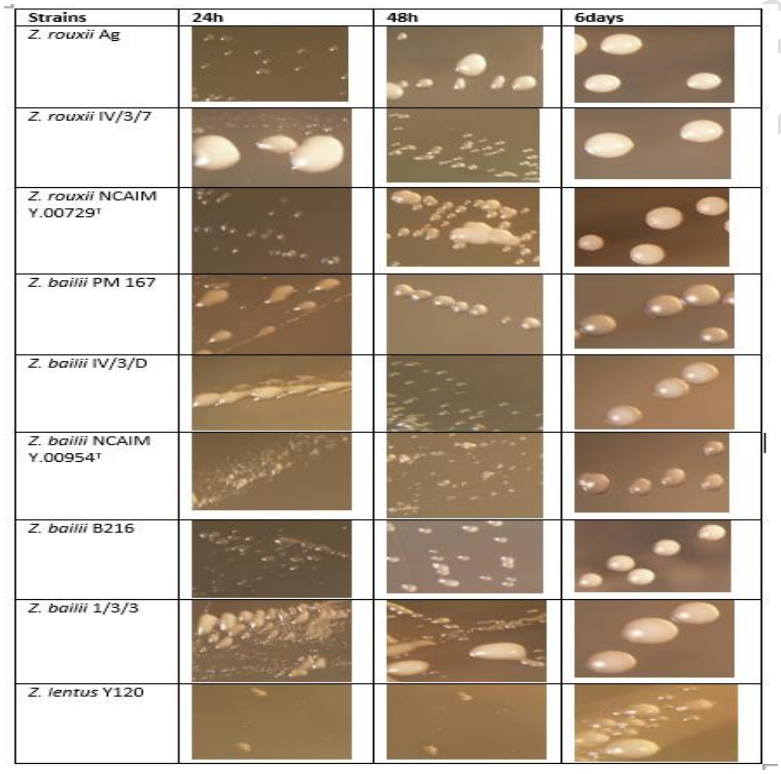


Figure 21. Images of colony morphologies of *Zygosaccharomyces* strains on modified M2 media

Results in Figure 21. show rapid growth rate in the modified M2 medium after changing the plating method and increasing the agar content from 1.5% to 3.0% w/v to solidify the culture medium (previously M2, in section 5.3.2, Fig. 16). There was observable positive growth in all the cases of *Zygosaccharomyces* strains except *Z. lentus* Y120 which showed a slow growth rate. According to Kurtzman and co-workers (2011), *Z. lentus* shows slow growth rate under aerobic conditions. This study also showed that *Z. lentus* Y120 grows well in semi-solid culture media with low viscosity to allow movement through the medium to access nutrients and water. It was also noted that media composition could contributed to the slow growth rate of some species.

5.4.2. Colony morphology of *Zygosaccharomyces* species on modified M3 agar at 25 ^{o}C

Results of colony morphologies of *Zygosaccharomyces* species on modified M3 agar at 25 ^{o}C after 24 h, 48 h, and 6 days of incubation scanned by Olympus SZX7 Stereo microscope and documented by DP10 camera are shown in Fig. 22.

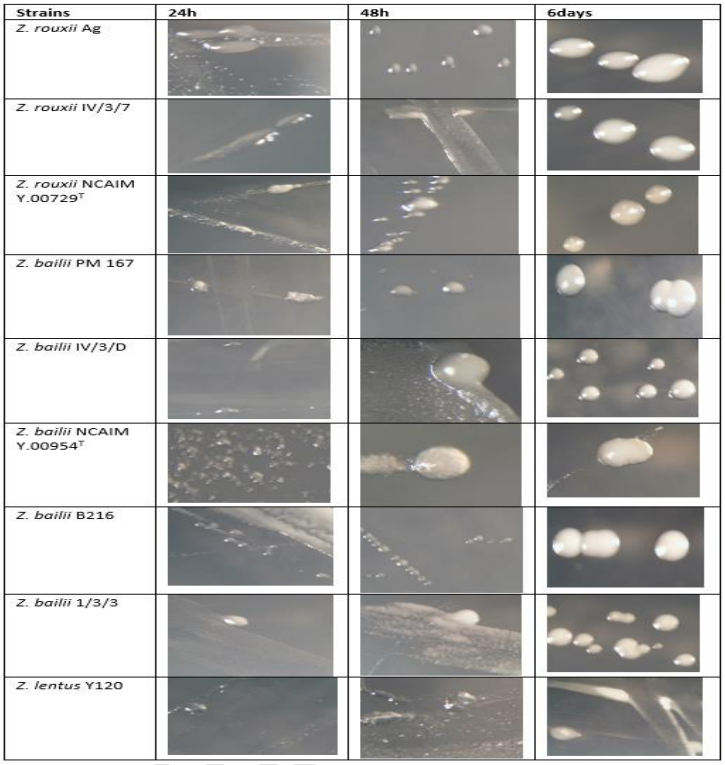


Figure 22. Images of colony morphologies of *Zygosaccharomyces* species on modified M3 medium

The results in Figure 22 show that there was poor growth of *Zygosaccharomyces* species after 48h of incubation on M3 agar. This was observed after increasing the agar concentration from 1.5% to 3.0% w/v. I hypothesised that increase of agar could have contributed to shear force leading to moisture lost thus reducing water activity (a_w) for optimal growth of the *Zygosaccharomyces* species. A previous study also reported the influence of agar content on the colony growth (Stecchini et al., 2001). However, it would be necessary to investigate further to determine the specific reason for poor growth of *Zygosaccharomyces* species in this particular medium.

5.4.3. Colony morphology of *Zygosaccharomyces* species on Modified M2[a] agar at 25 ^{o}C

Results of morphological characteristics of the nine *Zygosaccharomyces* strains tested on modified M2 (M2[a]) agar at 25 ^{o}C for 24 h, 48 h & 6 days listed in Table 9. (Olympus SZX7 Stereo microscope DP10 camera)

Table 10. Result of incubation of nine *Zygosaccharomyces* strains used in the study inoculated on modified M2 agar.

Strains	24h	48h	6days
Z. rouxii Ag	-ve	-ve	-ve
Z. rouxii IV/3/7	-ve	-ve	-ve
Z. rouxii NCAIM Y.00729^{T}	-ve	-ve	-ve
Z. bailii PM 167	-ve	-ve	-ve
Z. bailii IV/3/D	-ve	-ve	-ve
Z. bailii NCAIM Y.00954^{T}	-ve	-ve	-ve
Z. bailii B216	-ve	-ve	-ve
Z. bailii 1/3/3	-ve	-ve	-ve
Z. lentus Y120	-ve	-ve	-ve

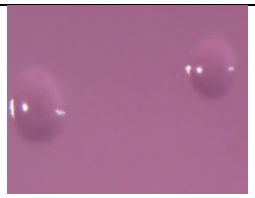
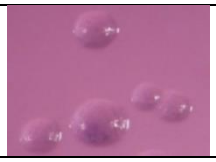
*-ve means no colonies observed on the culture medium with 0.5% of glacial acetic acid

There were no observable colonies on culture medium agar with 0.5% w/v glacial acetic acid as it is shown in Table 9. This is due to suppression effect of weak acid that inhibits the growth of yeast by acidification of the cytoplasm (Arneborg et al., 2000; James & Stratford, 2003; Palma et al., 2018). A previous study has also reported the inhibition of yeast species by acetic acid. The author disputed the assumption of co-resistance to weak acid preservatives, which has contributed to all weak acids having similar mechanisms of action, and that the mechanisms of resistance will protect the yeast cells against all weak-acid preservative (Steels et al., 2002) The acid molecules dissociate at near-neutral pH of the cytoplasm, releasing protons that causes the cytoplasm to become acidic, thus inhibiting the growth of the cells (Palma et al., 2017, 2018; Steels et al., 2002).

5.4.4. Colony morphology of *Zygosaccharomyces* species on modified $M2^b$ agar at 25 ^oC

Results of difference between the scanned macro morphological characteristics of nine *Zygosaccharomyces* strains tested on modified $M2^b$ agar at 25 ^oC for 24 h, 48 h & 6 days of incubation are shown in Table 10 and Fig. 23-25 (Stereo Microscopic picture with Olympus DP 10 camera)

Table 11. Images of colony morphological difference of *Z. bailii* and *Z. rouxii* strains on modified $M2^b$ agar.

Strains	**Modified $M2^b$**	**Strains**	**Modified $M2^b$**
Z. bailii B216		*Z. rouxii* Ag	
Z. bailii 1/3/3		*Z. rouxii* IV/3/7*(*)*	
Z. bailii PM 167		*Z. rouxii* NCAIM Y.00729^T	
Z. bailii IV/3/D		*Z. lentus* Y120	**No colonies**
Z. bailii NCAIM Y.00954^T			

Note: (*) misidentified at the isolation point

To get a clear difference between *Z. rouxii*, *Z. bailii*, and *Z. lentus* macroscopic morphology, bromophenol blue indicator, (which changes the colour from yellow at pH 3.0 and blue at pH 4.6) was added to a modified M2**b** without glacial acetic acid. Yeasts strains have different pH preferences and acid production (Kurtzman et al., 2011), which could have contributed to a noticeable difference in the colour of the colonies (**Table 11**). The center of colonies of *Z. rouxii* strains was darker as compared to *Z. bailii* strains due to the production of melanin pigment by *Z. rouxi* (Ali et al., 2020; Andreu et al., 2022; Petruzzi et al., 2017), while *Z. bailii* does not produce this metabolite (Gianvito et al., 2022). The results in Table 11. show difference in the colour of *Z. rouxii* IV/3/7 with other investigated *Z. rouxii* strains, while, there was a clear similarity of *Z. rouxii* IV/3/7 and the tested *Z. bailii* strains. This raised the suspicion that *rouxii* IV/3/7 was not a *Z. rouxii* but a *Z. bailii* strain.

Figure 23. Image of *Z. lentus* on modified M2^{b} agar (Phone Camera)

There was no observable growth of *Z. lentus* Y120 on the M2**b** agar with bromophenol blue as it can be seen in **Figure 23**. *Z. lentus* is sensitive to acidic conditions, if the pH of the medium with bromophenol is acidic, it could inhibit the growth of *Z. lentus* (Zuehlke et al., 2013). It is also possible that there were other factors that inhibited the growth of *Z. lentus* on this particular medium. Further investigation would be beneficial to determine the exact cause of the lack of growth of *Z. lentus* on the agar medium with bromophenol blue indicator. In addition, *Z. lentus* Y120 did not show a substantial positive growth in M2. It was also noted that no clear visible colonies of *Z. lentus* Y120 on modified M3 medium in Figure 22. as well as slow growth with no clear visible colonies on modified M2 medium as shown in Figure 21.

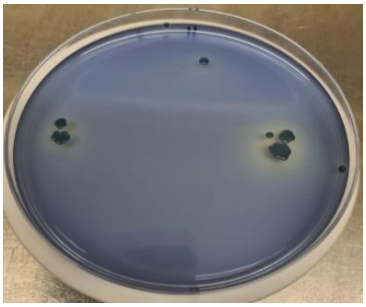


Figure 24. Image of *Z. rouxii* on modified M2^{b} agar *(*Phone Camera)

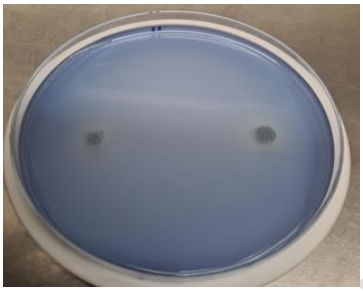


Figure 25. Image of *Z. bailii* on modified M2^{b} agar *(*Phone Camera)

The medium around *Z. rouxii* colonies in Figure 24. turned dark yellow, which indicates that the pH of the medium decreased due to the production of acidic metabolites by *Z. rouxii,* unlike the medium around *Z. balii* in Figure 25. which shows weak yellow or remained blue observed in other *Z. bailii* strains. This indicates that the pH of the medium did not change significantly as *Z. bailli* did not produce as much acid as *Z. rouxii* (Ali et al., 2020). Most of the strains showed similarities, but some performed slight differences in the magnitude of the colour of the colonies and the medium around the colonies, indicating strain variation (Péter, 2022). The results of this study are direct and noticeable consequences of fermentative ability of spoilage yeasts (James & Stratford, 2003; Loureiro & Malfeito-Ferreira, 2003; Sá-Correia et al., 2014).

5.4.5. Colony morphology of *Zygosaccharomyces* species on BSA culture media at 25 ^{o}C

Result of scanned macro morphological characteristic of *Z. lentus* Y120 tested on Bismuth Sulfite Agar at 25 ^{o}C after 7 days of incubation can be seen in Fig. 26.

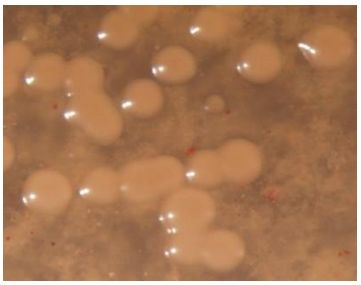
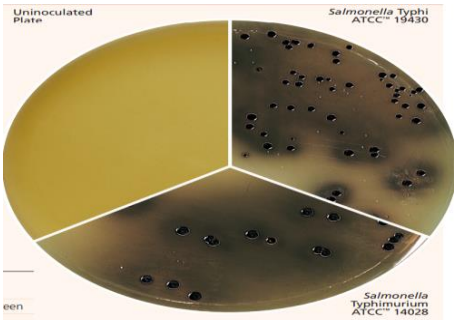


Figure 24. Result of *Z. lentus* Y120 on Bismuth Sulfite Agar media

Colony growth characteristics in **Figure 26** show that *Z. lentus* were raised smooth, round, convex and shinny colonies on BSA plates

It was noted that *Z. bailii* and *Z. rouxii* strains could not grow on the BSA culture media, which is a highly selective and differential medium for isolation of *Salmonella* species (Yanestria et al., 2019).



Becton Dickinson

PDF

Figure 25 Image of expected result of *Salmonella typhi* on BSA *273300, Becton Dickinson PDF*

My results confirm that *Z. lentus* can be differentiated from *Z. bailii* and *Z. rouxii* strains based on its positive growth on Bismuth Sulfite Agar medium at 25 ^{o}C after 7 days of incubation, as all other strains were not able to propagate their cells on BSA.

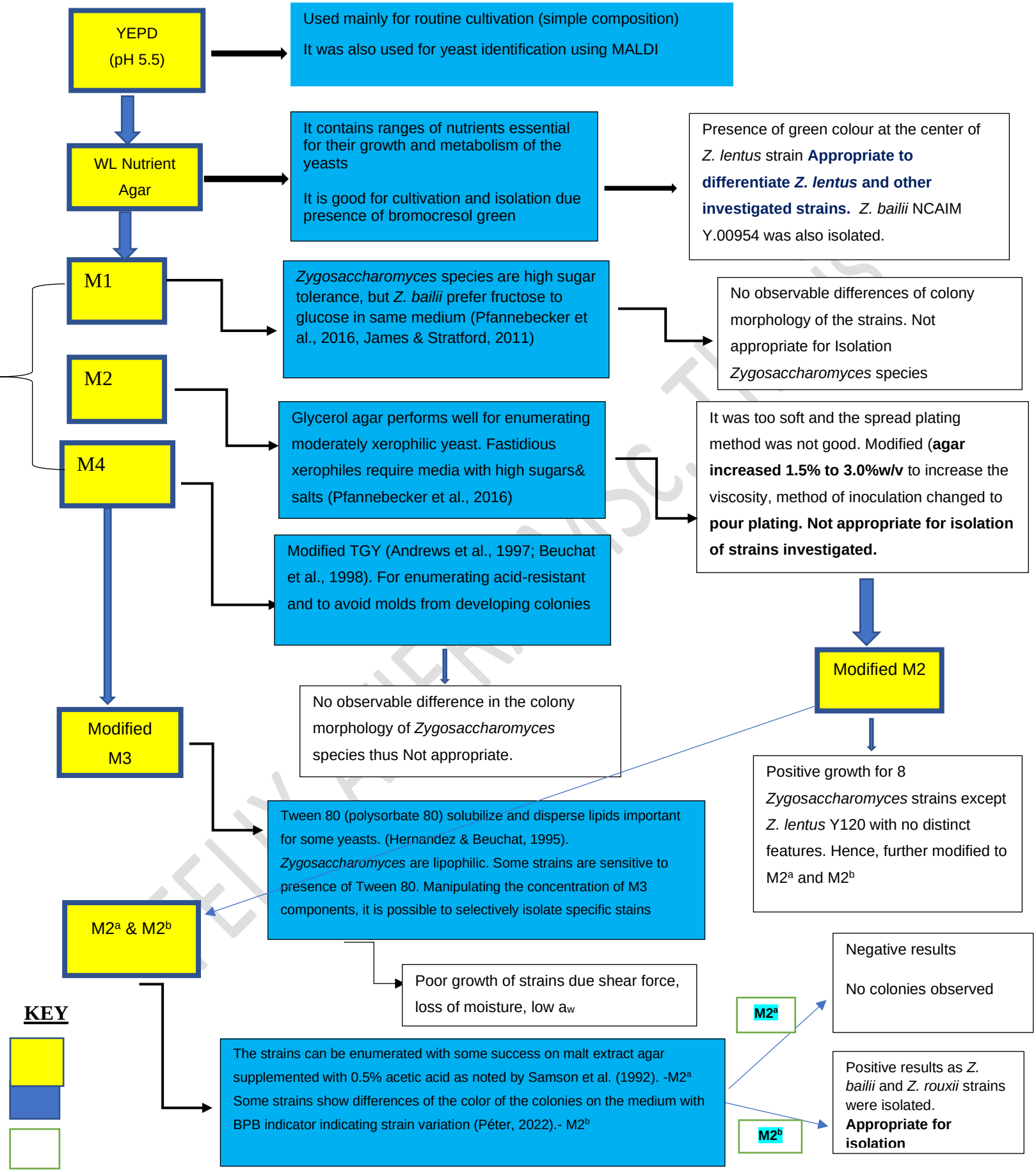
Flow diagram with sequential relationship of the choice of the media and outcome and/or conclusion is illustrated in page 40

Keynote:

Yellow- Culture media used for the investigation

Blue- The reasons for the choice of media to be tested for isolation of *Zygosaccharomyces* species

White- The outcome/ conclusion/ findings of the study


YEPD
(pH 5.5)
Used mainly for routine cultivation (simple composition)
It was also used for yeast identification using MALDI
WL Nutrient Agar
It contains ranges of nutrients essential for their growth and metabolism of the yeasts
It is good for cultivation and isolation due presence of bromocresol green
Presence of green colour at the center of Z. lentus strain Appropriate to differentiate Z. lentus and other investigated strains. Z. bailii NCAIM Y.00954 was also isolated.
M1
Zygosaccharomyces species are high sugar tolerance, but Z. bailii prefer fructose to glucose in same medium (Pfannebecker et al., 2016, James & Stratford, 2011)
No observable differences of colony morphology of the strains. Not appropriate for Isolation Zygosaccharomyces species
M2
Glycerol agar performs well for enumerating moderately xerophilic yeast. Fastidious xerophiles require media with high sugars& salts (Pfannebecker et al., 2016)
It was too soft and the spread plating method was not good. Modified (agar increased 1.5% to 3.0%w/v to increase the viscosity, method of inoculation changed to pour plating. Not appropriate for isolation of strains investigated.
M4
Modified TGY (Andrews et al., 1997; Beuchat et al., 1998). For enumerating acid-resistant and to avoid molds from developing colonies
Modified M2
No observable difference in the colony morphology of Zygosaccharomyces species thus Not appropriate.
Modified M3
Positive growth for 8 Zygosaccharomyces strains except Z. lentus Y120 with no distinct features. Hence, further modified to M2a and M2b
Tween 80 (polysorbate 80) solubilize and disperse lipids important for some yeasts. (Hernandez & Beuchat, 1995). Zygosaccharomyces are lipophilic. Some strains are sensitive to presence of Tween 80. Manipulating the concentration of M3 components, it is possible to selectively isolate specific stains
M2a & M2b
Poor growth of strains due shear force, loss of moisture, low aw
KEY
The strains can be enumerated with some success on malt extract agar supplemented with 0.5% acetic acid as noted by Samson et al. (1992). -M2a Some strains show differences of the color of the colonies on the medium with BPB indicator indicating strain variation (Péter, 2022).- M2b
M2a
Negative results
No colonies observed
M2b
Positive results as Z. bailii and Z. rouxii strains were isolated. Appropriate for isolation

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusions

- The result of this study show that yeast suspension with modified culture medium M2^{b} (Agar, 1.5%w/v, Yeast extract 0.5%w/v, Glucose 30% w/v, Glycerine 10% w/v, KCl 10% w/v & Bromophenol blue indicator 0.002% w/v) is appropriate to differentiate between *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* species based on pH change during the growth and production of melanin associated with *Zygosaccharomyces rouxii* However, different strains have different physiological properties and metabolic capabilities.

- It was also noted that WL Nutrient agar could be used to differentiate between *Zygosaccharomyces lentus* (Y120) and other *Zygosaccharomyces* species (*bailii* and *rouxii*) attributed by the bromocresol green indicator due to the pH change during the growth on the umbonate colonies of *Z. lentus* Y120

- Certain culture media and their composition may also influence the growth, recovery and isolation certain *Zygosaccharomyces* species than others. Results of modified M2^{a} medium with 0.5% glacial acetic acid showed no colonies of *Zygosaccharomyces* strains. In addition, *Z. lentus* Y120 strain either did not grow or not performed well on culture media with glycerine (10-18%w/v) especially on M2, M3, Modified M2^{a} & M2^{b} except M1, M4, BSA and WL Nutrient agar with no glycerine, (these media supported its growth). Therefore, the choice of media should be based on the specific *Zygosaccharomyces* species being targeted.

- Our study result revealed that *Zygosaccharomyces lentus* Y120 could grow on Bismuth Sulfite Agar which is a selective medium for the isolation of *Salmonella Typhi*. Similarly, to *Candida* species which have the ability to reduce sulphite to sulphide giving a brown to black colony *colour, Z. lentus* is able to convert this ingredient and utilize the other component of BSA. It was suggested that further research should be done on this particular strain.

6.2. Recommendations

- Based on the conclusions drawn from testing of different culture media for isolation *Zygosaccharomyces* species, further testing of different culture media, would be useful to determine whether there are any more effective or selective media for the growth and isolation of specific *Zygosaccharomyces* species.

- I also suggested for testing for more yeast (mainly *Zygosaccharomyces*) isolates/strains from different origin to be able to determine the efficiency of the culture media used in this study. As an important result it was possible to prove the fact of wrong identification in the case of *Z. rouxii* IV/3/7; it had been misidentified at the collection point.

7. SUMMARY

Zygosaccharomyces species are important yeasts commonly found in fermented food and beverage. However, they are also known as the most troublesome food spoilage organisms in the high-salt and high-sugar food and beverage industries. Consequently, there has been a need for new and improved media for selectively isolating various *Zygosaccharomyces* species and/or strains. The main objective of this study was to test different culture media for the isolation of *Zygosaccharomyces* species. Therefore, 10 different culture media (YEPD, WL Nutrient agar, M1, M2, M4, Modified M2 & M3, Modified M2$^{\mathbf{a}}$ & M2$^{\mathbf{b}}$, and BSA) were prepared and tested against nine *Zygosaccharomyces* strains: *Z. rouxii* Ag, *Z. rouxii* IV/3/7, *Z. rouxii* NCAIM Y.00729^{T}, *Z. bailii* PM 167, *Z. bailii* 1/3/3, *Z. bailii* IV/3/D, *Z. bailii* B216, *Z. bailii* NCAIM Y.00954^{T} and *Z. lentus* Y120). The MALDI-TOF MS (Brucker's Flex series) was used for the identification of the yeasts, while an Olympus SZX7 Stereo microscope and Olympus BX41 Fluorescence microscope was used for macro- and micromorphological examination of the strains, respectively. Results for *Zygosaccharomyces* identification showed a match with low confidence identification of a Z score of 1.86 and 1.87 for *Z. bailii* strains, while in the cases of the other strains there were either no peak found or no organism identification was possible with a Z-score between 0.00-1.65. However, the database used for identification was developed for clinical application, thus we could see the software was not appropriate for my purpose. The results for colony morphology characteristics showed that WL Nutrient agar is appropriate to differentiate the *Z. lentus* from other investigated *Zygosaccharomyces* species due to the presence of bromocresol green indicator that turns green at the center of *Z. lentus* strain giving the colonies with specific characteristics. The results on the M1 medium showed a steady growth of colonies of *Zygosaccharomyces* strains with no distinct features, while the results on M2, modified M2 and M3, M2^{a} and M2^{b} media with 10-18% glycerine had a strong correlation specifically for *Z. lentus* Y120 which either did not growth on the media, slow growth, or growth with no clear visible colonies. Results for M2^{b} medium with bromophenol blue indicator showed a positive difference in colony morphology of *Z. bailii* and *Z. rouxii* strains. BSA medium showed positive growth for *Z. lentus*, while the remaining *Zygosaccharomyces* strains had negative responses. Therefore, this study revealed that WL Nutrient agar is appropriate for isolation of *Z. rouxii* strains, while M2^{b} for isolation between *Z. bailii* and *Z. rouxii* strains.

KEYWORDS

Food & beverage spoilage, *Zygosaccharomyces* strains, Culture media, Identification & isolation

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9. APPENDICES

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