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Enhancing Beverage Fermentation through Synergy of *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae*: A Mini-Review

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Authors' contributions

This work was carried out in collaboration between both authors. The author BS has conceptualized the manuscript, prepared the first draft of manuscript and reviewed the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Microbial fermentation; a natural process dating back over 7000 years BC, plays a pivotal role in beverage production. While *Saccharomyces cerevisiae* dominates the industry, recent research emphasizes the importance of co-culture with non-*Saccharomyces* yeasts for enhanced flavor and aroma. This review explores the cooperative interaction between *Saccharomycopsis fibuligera* and *S. cerevisiae* in alcoholic fermentation, shedding light on their enzymatic capabilities. *S. fibuligera*, an ascomycete with potent amylolytic activity, demonstrates the ability to efficiently convert starch into alcohol, contributing to improved fermentation stability. Co-culturing with *S. cerevisiae* unleashes a biochemical diversity that enhances the sensory attributes of beverages. Beyond flavor

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complexity, the co-culture strategy influences key compounds, including phenolic compounds and esters, elevating overall quality. The review delves into the biochemical intricacies of starch-based fermentation, emphasizing the potential of *S. fibuligera* in hydrolyzing starch into fermentable sugars. *S. cerevisiae*, a versatile and genetically diverse yeast, adapts to different environmental conditions crucial for successful fermentation. The co-culture approach not only accelerates fermentation but also combats contamination and reduces overall processing time.

Keywords: S. fibuligera; S. cerevisiae; co-culture; fermentation.

1. INTRODUCTION

1.1 Importance of Co-Culture in Beverage Fermentation

Microbial fermentation is a natural process during which larger organic molecules are broken into simpler ones [1]. The earliest archaeological evidence of fermented beverage production technology dates back to over 7000 years BC in China. This evidence was found in ancient tombs and is associated with the production of fermented beverages based on rice, honey, and fruit. Chemical analysis of data confirming the beginning of wine making dates back to 5400 BC, indicating the ancient origins of fermented beverage production [2]. The wine or beer-making process includes complex sugar molecules, dominantly starch of fruit and cereal origins which are converted to fermentable sugars through spontaneous fermentation by natural microbiota. The use of carefully selected commercial yeast strains has significantly improved the controllability and reliability of the wine fermentation process, limited variability in wine microbial composition, and made a significant contribution to the improvement of wine quality in recent decades. Moreover, Controlled fermentation debarred the risk of otherwise stuck fermentation, spoilage contamination and a relatively undesired final aroma, in spontaneous fermentation [3]. This widespread application of yeast in wine making has pushed these microorganisms to the forefront of genetic, genomic, and biochemical research, leading to a deeper understanding of their impact on wine characteristics and the development of novel strategies for targeted selection and creation of novel strains to improve wine making conditions [2].

In the beverage industry, *Saccharomyces* yeasts, including top-fermenting *S. cerevisiae* and bottom-fermenting *S. carlsbergensis*, are extensively utilized for the production of beer and wine [4]. These yeasts have been domesticated and tailored for specific brewing processes to achieve desired flavor profiles and fermentation outcomes. The Predominance of *S. cerevisiae* over other genera is widely reported [5], however, there is an increase interest in the role of non-*Saccharomyces* yeasts in mixed inoculated fermentation for the betterment of the chemical and sensory properties of wine [5,6]. This approach involves the co-fermentation of non-*Saccharomyces* yeast and *S. cerevisiae*, leveraging the rich enzymatic capabilities of non-*Saccharomyces* yeasts to hydrolyze and release abundant aroma compounds [7]. Additionally, co-fermentation with non-*Saccharomyces* yeasts such as *Issatchenkia terricola*, *Pichia kudriavzevii*, and *Metschnikowia pulcherrima*, *Saccharomycopsis fibuligera* which possess high β-glucosidase activity, has shown promising results in improving the overall sensory attributes of wines [6,8,9].

In the last few years, wine researchers have explored the controlled use of non-*Saccharomyces* yeasts along with *S. cerevisiae* strains [5,6,10,11]. Through simultaneous and sequential inoculation fermentation methods; the synergistic effects of co-culturing these yeast strains with *S. cerevisiae*, paves the way for innovative strategies to optimize alcohol fermentation processes. These co-culture approaches not only offer the potential to enhance flavor complexity and aroma characteristics, it can also modulate the expression of other important compounds such as phenolic compounds, terpenes, acetate, and ethyl esters, which greatly contribute to the quality and sensory properties of wines. In this mini-review, the attributes of *S. fibuligera* and *S. cerevisiae* leading to enhanced fermenting products are elaborated to give a gist of the advantages of co-culture of these two otherwise industrially important yeasts.

1.2 Understanding the Process of Alcoholic Fermentation

The wine or beer industry, like any other bioethanol formation, depends on the conversion

of carbohydrate macromolecules to alcohol. However, in wine or beer fermentation the raw material is starch from fruit or cereal origin, utilized by yeasts and bacteria. The role of yeast in alcoholic fermentation was established by Louis Pasteur in 1860 [2]. His work laid the foundation for our understanding of the crucial role that yeast plays in the fermentation processes involved in the production of alcoholic beverages. Starch-based fermentation consists of two main steps: Starch liquefication and saccharification followed by the conversion of fermentable sugars to ethanol [8]. *S. cerevisae* mediated alcoholic fermentation involves a series of complex biochemical reactions that contribute to the conversion of fermentable sugars into alcohol and other compounds. In the case of co-culture fermentation with non-*Saccharomyces* yeasts and *S. cerevisiae*, the biochemical processes are even more diverse and intricate.

Starch, the primary substrate of alcoholic fermentation, is a polymer of glucose and mainly consists of amylose and amylopectin. Amylose is mostly a linear molecule containing α-d-glucosyl units that are essentially linked by α-1,4-glycosidic bonds, whereas amylopectin is a highly branched structure composed of large polymers of α-1, 4-glycosidic bonds linked α-d-glucosyl units with α-1, 6-linked side chains. The α-1, 4-glycosidic linkages and branched α-1, 6-glycosidic linkages are hydrolyzed by the enzymes alpha-amylase, an endo-acting enzyme, and glucoamylase, an exo-acting enzyme [12]. There are two possibilities for microbial utilization of starch: (1) to use a microbial strain, either constructed by genetic engineering techniques or selected from a natural source, which can hydrolyze starch and ferment sugars to ethanol; or (2) to divide the process into two separate steps: starch hydrolysis and ethanol formation. In the later process, the starch hydrolysis stage is usually performed by free, immobilized or co-immobilized systems of commercial amylolytic enzymes or directly by an amylolytic microbial strain. Subsequently, a specific non-amylolytic strain is used for product/ethanol formation [13]. The second possibility focusing on the role and attributes of *S. fibuligera* as an amylolytic agent and *S. cerevisiae* as the fermenting agent is of prime interest [14].

The alcoholic fermentation process initiated by *S. cerevisiae* and non-*Saccharomyces* yeasts involves multiple enzymatic activities. Non-*Saccharomyces* yeasts, such as *S. fibuligera, Issatchenkia terricola, Pichia kudriavzevii,* and *Metschnikowia pulcherrima*, are rich in various enzymes, including α-amylase, glucoamylase and β-glucosidase, that play crucial role in the fermentation process [10,15,16]. The action of alpha-amylase leads to the release of maltose, smaller oligosaccharides, and dextrin as the main products. However, the action of glucoamylase from the non-reducing ends of starch chains leads to the production of glucose. Furthermore, these enzymes catalyze the hydrolysis of glycosidically bound aroma compounds, releasing volatile aroma compounds, such as terpenes and esters, contributing to the enhanced sensory attributes of wines [17]. As mentioned earlier, co-fermentation of non-*Saccharomyces* yeasts with *S. cerevisiae* leads to the modulation of important compounds such as phenolic compounds, terpenes, acetate, and ethyl esters. These compounds greatly influence the quality and sensory properties of wines, enhancing the overall flavor complexity and aroma characteristics. The expression of different enzymatic activities and the collaborative metabolic processes between non-*Saccharomyces* yeasts and *S. cerevisiae* in co-culture fermentation contribute to the unique biochemical profile of the final product. This biochemically diverse environment, brought about by the co-culture of yeasts, sustains the production of a wide array of compounds that are essential for the overall sensory attributes and complexity of wines [8].

1.3 *Saccharomycopsis fibuligera*

Saccharomycopsis fibuligera, also known as *Endomyces fibuliger* or *Saccharomyces fibuligera*, is an ascomycete that has been identified as the main amylolytic yeast in fermenting foods, particularly in rice wine fermentation [18]. It is a dimorphic yeast with round or oval cellular morphology that can form mycelia by budding (Fig. 1). The ascocarp of *S. fibuligera* are oval and free individual vegetative cells that adhere to the end or side of the mycelium [19]. The surface of *S. fibuligera* colony is white and rough (Fig. 1). Wickerham et al. [20] were the first to report on starch hydrolysis by *S. fibuligera*, which laid the foundation for the Swedish Symba yeast process. *S. fibuligera* is a potential industrial microorganism, as it shows low requirements for culture medium, strong adaptability and fast growth [19]. *S. fibuligera* has high acid protease, β-glucosidase, glucoamylase, and α-amylase activities, thus,

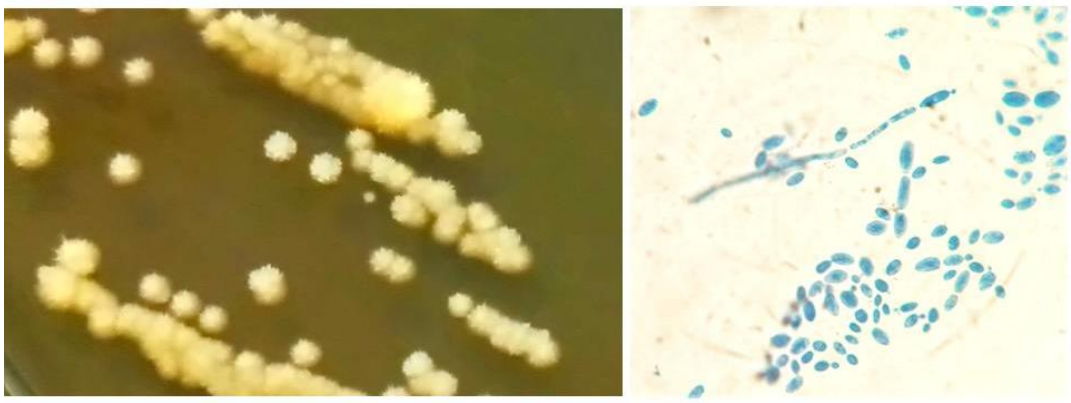

Fig. 1. Cultural and morphological characters of *Saccharomycopsis fibuligera* on Potato dextrose Agar and under 40x of compound microscope [19]

it can use starch to synthesize trehalose and use starch and protein to synthesize cell proteins, as a protein feed [21,22,23]. The ability of *S. fibuligera* to degrade native starch into dextrin, maltose, and glucose through the production of various metabolic intermediates makes it an invaluable tool in various industries including food, fermentation, biofuel, and pharmaceutical. In the wine or beer industry, *S. fibuligera* can generate aromas and esters, which can improve the quality of liquor. Furthermore, due to its high saccharification and relatively low fermentative ability, *S. fibuligera* can also be used solely to produce low alcohol containing beer [24]. In the fermentation industry, *S. fibuligera* improves the overall quality of liquor through four major contributions, firstly, by its high starch conversion capacity, which enables it to efficiently convert starch into alcohol during the fermentation process under appropriate pH conditions. The optimal pH range for alpha-amylase activity is typically around 5.0 to 7.0, although this can vary depending upon the specificity of the enzyme produced by *S. fibuligera* [14]. Secondly, the production of various aromas and esters during the fermentation process, contributes to the flavor and aroma of the final sensory characteristics of the product [14]. Thirdly, *S. fibuligera* is more tolerant to low-temperature stress than other yeasts, making it valuable in low-temperature liquor fermentation. This property improves the stability of volatile compounds, reduces evaporation loss and resulting in a higher-quality product [14]. Fourthly, co-fermenting *S. fibuligera* with other strains produces a more complex flavor profile and improve the overall quality of the final product. For example, in the production of Xiaoqu liquor, inoculating functional yeast such as *S. fibuligera* and *S. cerevisiae* can improve the alcohol and ester content of the final product [14].

S. fibuligera has been isolated from several traditional cereal-based alcoholic beverages all around the globe including Xiaoqu [25], Daqu [3], Nuruk [26], Dombea [26] and Xaaj [19], basically fermented individually from rice, wheat, barley, sorghum, others grains, pea and their combinations.

1.4 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, an ascomycetous yeast (Fig. 2), has been an essential component of human civilization and has been extensively studied due to its importance in the production of fermented beverages such as wine, beer, and bread. In the European yeast industry, 1 million tonnes is produced annually, and around 30% of which is exported globally [27]. The global market's annual growth rate was 8.8% from 2013 to 2018. Regarding the beverage industry, *S. cerevisiae* is involved in the production of many fermented beverages, such as wine, beer and cider; distilled beverages, such as rum, vodka, whisky, brandy, and sake; in other alcoholic beverages worldwide, from fruits, honey, and tea, *S. cerevisiae* is also involved. Fermentation can take place either from a spontaneous development of the raw material's microflora or from the addition of a pure yeast culture. A discussion on the contribution of *S. cerevisiae* in wine, bread and cocoa fermentations follows, highlighting aspects such as the biochemical reactions that take place in the cell and whose products determine the final products, the traits that strains must have to be successful starters and the potential of exploiting native strains in the industry [27].

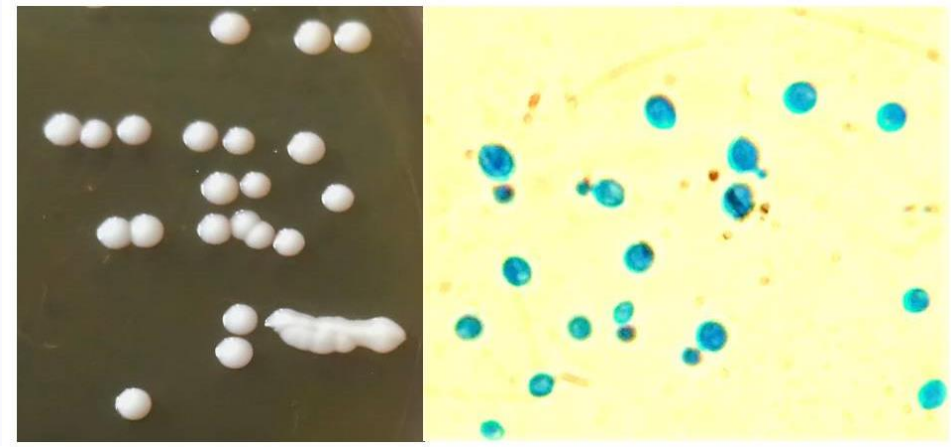

Fig. 2. Cultural and morphological characters of *Saccharomyces cerevisiae* on Potato dextrose Agar and under 40x of compound microscope [19]

Some of the biochemical peculiarities of *S. cerevisiae* that make it the sole player in the fermentation industry is as follows. Ability to ferment glucose and other sugars to produce ethanol and carbon dioxide; ability to tolerate high levels of ethanol, which is important for the production of alcoholic beverages; production of various flavor and aroma compounds during fermentation, including esters, higher alcohols, and sulfur-containing compounds; Ability to synthesize and secrete enzymes such as amylases and proteases, which are important for the breakdown of complex carbohydrates and proteins during fermentation. Most importantly, it can adapt to different environmental conditions, including changes in temperature, pH, and nutrient availability, which is important for its survival in various fermentation environments [2].

The genetic variability of *S. cerevisiae* contributes to its biochemical adaptability by allowing the selection of strains with specific traits that are desirable for industrial applications, such as the production of fermented beverages. The specific traits arise from natural genetic variation, as well as from the artificial selection of strains with specific traits. Studies have shown that *S. cerevisiae* strains from different geographic regions and ecological niches exhibit significant genetic and phenotypic diversity [2]. This diversity is thought to arise from a combination of factors, including genetic recombination, mutation, and selection. In addition to natural genetic variation, artificial selection has also played a significant role in shaping the genetic diversity of *S. cerevisiae*. For example, the selection of strains with specific traits, such as high ethanol tolerance or efficient sugar utilization, has led to the development of commercial yeast strains that are widely used in the production of fermented beverages [2].

1.5 Co-culture of *S. fibuligera* and *S. cerevisiae*

Yeast-yeast interactions enhance the aromatic profile of alcoholic beverages [28]. The use of non-*Saccharomyces* species as starters, along with various *S. cerevisiae* strains improved considerably various wine characteristics, such as physicochemical properties, the composition and concentration of the wine's volatile compounds, *i.e*. flavour, aroma of the final product, glycerol concentration and others. Ciani and Comitini [29] and Parapouli et al. [27] have discussed the co-culture of *Saccharomyces* with non-*Saccharomyces* yeasts of the genera *Candida, Debaryomyces, Hanseniaspora, Tolurospora, Metschnikowia, Wickerhamomyces* etc.

There are several benefits of using controlled co-culture fermentations with more than one selected yeast strain in wine making. Mixed fermentations can lead to changes in the levels of specific compounds such as glycerol, total acidity, and acetic acid, which can impact the overall analytical profile of the wine. The use of controlled multi-starter fermentation with selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeast strains can lead to an enhancement of the aroma profile of the wine, including the production of esters and volatile thiols, contributing to the overall complexity of the wine. Multi-starter fermentations have the potential to reduce the ethanol content of the wine, which can be a desirable trait for certain

wine styles. The use of mixed fermentations can help in controlling the presence of undesirable microorganisms in the wine, contributing to improved overall quality and complexity.

Yeast interactions in co-culture fermentation can affect metabolite production and microbial growth in the fermentation process. For example, the production of medium-chain fatty acids and high amounts of acetic acid can negatively affect the growth of a co-fermenting yeast species [29]. Additionally, cell-to-cell contact appears to be involved in the interactions between *S. cerevisiae* and other non-*Saccharomyces* species. Reduced oxygen availability under grape juice fermentation might have an important role as a selective factor in mixed cultures. Furthermore, different yeast strains in co-cultures can have positive or negative interactions regarding different analytical compounds. For example, continuous culture of *S. fibuligera* and *Candida utilis* gives positive interactions while applied one after another [30]. In some cases, the aromatic profile of the wine is influenced by the simple addition of metabolites produced by each yeast from partial consumption of carbon or nitrogen sources, or by a specific metabolic activity.

Co-culture of amylolytic *S. fibuligera* and fermentative *S. cerevisiae* provide reducing sugars, by action of an amylolytic agent on starch, for conversion into ethanol through anaerobic fermentation by the fermentive agent. Research related to fermenting wine using these two yeasts is limited. Recently, Su et al. [15] attempted to improve the flavour profile of Xiaoqu liquor, by selecting these two functional yeast strains (*S. fibuligera* and *S. cerevisiae*) for Xiaoqu liquor fermentation. Results found that compared with traditional Xiaoqu, bioaugmentation inoculation increased the glucoamylase, acidic protease activities and the ethanol synthesis rate while decreasing acidity during the early stage of fermentation. By the end of the fermentation process, the alcohol and ester content had also increased by 42.5% and 11.8%, respectively, and that of aldehydes and ketones, and heterocyclic compounds decreased by 73.7% and 77.1%, respectively. Inoculation of *S. fibuligera* and *S. cerevisiae* into Xiaoqu increases the activity of Xiaoqu liquor protease and saccharifying enzyme, alters the diversity of bacteria, and increases the alcohol and ester content [15]. This was well explained as initial inoculation of *S. fibuligera* led to an increase in ethanol content explaining its role in early fermentation potential through enzymatic activity [13]. This amylolytic yeast contributes to the flavor, aroma and ethanol content in the early stage of fermentation. As fermentation progresses pH value changes making it unsuitable for the amylase activity of *S. fibuligera* fom where *S. cerevisiae* takes up the fermentation process.

Ongoing research efforts are focusing on exploiting the benefits of using *S. fibuligera* in fermentation, including, genetic engineering and breeding to introduce target genes into vectors to construct new recombinant strains with enhanced traits for improving the quality of industrial products.. Exploring the potential of *S. fibuligera* in low-temperature liquor fermentation to improve the stability of volatile compounds and reduce evaporation loss. Investigating the potential of *S. fibuligera* in reducing production costs in industrial production by efficiently adsorbing starch granules and hydrolyzing raw starch within a certain pH range. Further analysis of the functional characteristics of *S. fibuligera*, its role in the regulation of metabolic pathways associated with liquor fermentation and its relationship with other liquor microorganisms through genomic data and molecular biology research [14].

2. CONCLUSION

In conclusion, the co-culture of *Saccharomycopsis fibuligera* and *S. cerevisiae* holds significant promise for enhancing the fermentation process in the beverage industry, particularly in wine and liquor production. Leveraging the enzymatic capabilities of *S. fibuligera* in starch hydrolysis, coupled with the fermentative prowess of *S. cerevisiae*, offer a strategic approach to optimizing alcohol fermentation. This cooperative interaction not only enriches the flavor complexity and aroma characteristics of the final product but also modulates key compounds, such as phenolic compounds and esters, contributing to overall quality, reducing the time of fermentation and combating contamination. Research efforts, including genetic engineering and breeding, are underway to harness the full potential of *S. fibuligera* in improving the stability of volatile compounds and reducing production costs. The co-culture approach represents an innovative strategy in the ever-evolving landscape of beverage fermentation, showcasing the dynamic interplay between different yeast strains for the advancement of product quality and sensory attributes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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