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ISOLATION AND CHARACTERIZATION OF YEASTS FROM LOCALLY AVAILABLE FOODS

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ABSTRACT

A study was conducted to identify different isolates of yeasts which are prospective to be utilized in various industries from locally available foods. Altogether 24 yeast isolates were obtained from fermented fruits and vegetables (banana, cabbage, grapes, lime and mango), pudding, bee honey, toddy and fermented fish samples using the pour plate method. Characterization of the yeasts using several biochemical tests (urease, catalase, liquid carbon and nitrogen assimilation and sugar fermentation tests) revealed that this pool was composed with yeast strains belong to genera of Saccharomyces, Kluvyeromyces, Candida, Pseudozyma, Cryptococcus, Rhodotorula and Debaryomyces. The most effective lactose fermenters were identified as viable candidates for bioethanol production and for manufacturing fermented dairy products for lactose intolerant people. Yeasts with the highest biomass production were suggested as the best viable candidates for industrial single cell protein production using whey, the major byproduct of the dairy industry. The five thermo-tolerant yeasts (Y55, Y57, Y58, Y59 and Y70) and Y069 which was optimally active under 10C were recognized as suitable for industrial applications. The isolates tolerant for high osmotic pressure conditions were identified as potential isolates to be used in highly concentrated food products. Sugarcane juice was recognized as a possible medium for the cultivation of these yeasts in industrial settings. The beneficial yeasts forecasting in this study are expected to screen using molecular biological methods to utilize them in industrial applications.

Keywords: Characterization, Fermented foods, Isolation, SCP, Yeasts

INTRODUCTION

Yeasts are single celled eukaryotic organisms belonging mainly to the *Ascomycetes*, classified under kingdom Fungi. So far the total number of yeast species discovered is around 1500. Even though yeasts are found in many diverse environments, yeast species have highly specialized natural habitats and thus, it is possible to isolate specific strains from appropriate habitats. (Chandrasena *et al*., 2006). Studies conducted worldwide have revealed that many beneficial yeast strains are possible to be isolated from various fermented foods (Dubash *et al*., 2010; Gana *et al*.; 2014; Moreira *et al*., 2001; Obasi *et al* 2014). But, so far only a limited number of research studies have been carried out in Sri Lanka to isolate yeasts from locally fermented foods and to screen them for their beneficial properties in a broad way. Thus in this research study, the utmost attempt was to isolate maximum possible number of yeast types from a wide range of locally available foods and to identify their possible beneficial characteristics and industrial applications with the purpose of solving the most common burdens of several industries in Sri Lanka. For the identification of yeast, several biochemical tests were used along with the information available in previous literature and this study is expected to proceed with further molecular biological studies.

METHODOLOGY

Isolation and maintenance of yeast isolates

Fruits and vegetables (Grapes, Cavendish and Embul varieties of banana, mango, lime and cabbage), smoked fish, bee honey, toddy, yoghurt and pudding samples were collected from local market, Colombo, Sri Lanka for the isolation of yeast. Ten grams of each of the fruit and vegetable samples were stored in sterile stomacher bags under 25◦C allowing for fermentation. All samples were serially diluted ($10^{-1}$to 10^{-8}) with 0.85%Nacl solution. Yeasts in above dilutions were isolated and enumerated using pour plate technique. Yeast Potato Dextrose (YPD) medium was used for growing yeast. Incubation was done for 1-2 days at 25◦C. Maintenance of cultures was done following the method given by Obasi *et al* (2014) with some modifications. The discrete isolated colonies were purified by re-streaking on YPD plates and maintained on slants with 2% agar of the same medium at 5◦C in the refrigerator and as frozen stocks in liquid YPD medium with 40% glycerol at -20◦C.

Characterization of isolated yeasts

Catalase test

A small amount of colonies from each yeast isolate was transferred to clean, dry glass slides using a loop and a drop of 3% H_2O_2 placed on the slide and mixed. Rapid evolution of oxygen as evidenced by bubbling within 5-10 seconds was considered as the positive result.

Urease test

Urea hydrolysis test was conducted by preparing the urea agar base (Code: CM0053) at the laboratory as expressed by Christensen, 1946. Surface of the Urea agar slope was heavily inoculated with a pure culture of each yeast isolate to be tested. Conversion of the medium from orange to pink after storing at 35C for 3-5 hours was taken as positive reaction.

Liquid assimilation of carbon and nitrogen compounds

This was conducted by following the methodology used in the researches by Moeini *et al.*, 2004 and Nahvi and Moeini, 2004. The carbon compounds tested were sucrose, glycerol, raffinose, D-maltose, D-manitol and L-rhamnose. The nitrogen compounds tested were nitrate and L-lysine and L- Cysteine.

Sugar fermentation test

Ability to ferment sugars (lactose and sucrose) was tested following the method explained in Moeini *et al.*, 2004.

Screening for industrial applications

Ability to ferment in different temperature conditions

Fermentation of YPD broth (5 g yeast extract, 10 g peptone and 10g glucose per 1l of broth) under 10C and 45C temperature conditions was tested by using 0.04% bromocrysol purple stock solution as the indicator. Both acid and gas production during fermentation were evaluated.

Ability to ferment in sugarcane juice

Sugar cane juice of 45% distilled water on weight basis with Bromothymol blue was inoculated with yeast isolates and acid production was evaluated. Test tubes with 5ml of extract were incubated at 25C temperature for 7-10 days and color changes were recorded.

Ability to produce SCPs

Falken tubes with laboratory made whey were inoculated with actively growing yeast cultures and were stored in a shaking incubator at 25C temperature and 150rpm for 10 days. Produced protein was separated using filter method and SCP production was analyzed based on the bio mass production

Ability to grow in 50% glucose

Isolated yeast strains were inoculated in YPD medium with 50% glucose using single streaking method. After the incubation for 3-4 days their growth was analyzed.

RESULTS AND DISCUSSION

A total of 24 yeast isolates were obtained and identified according to their colony morphologies (Figure 1) and light microscopic views from the samples of locally abundant fermented food items.

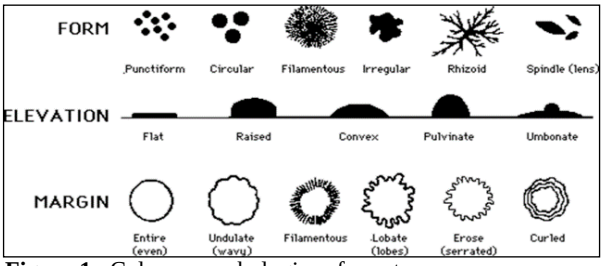

Figure 1 : Colony morphologies of yeast
Source: http://www.pinterest.com

For the isolation of yeast, peel was also used along with the pulp in all fruits and vegetables. Because, yeast prefers aerobic conditions for their growth. Being highly oxidative, peel of fruits and vegetables was identified as a good source to isolate yeast micro flora. Amongst the fermented food sources used for isolation of yeast, fermented grapes and banana have given the highest number of yeast isolates (Table 1).

Table 1 : Yeasts isolated from fermented foods

Food item	Number of yeast isolates
Fermented grapes	4
Fermented mango	2
Fermented cabbage	1
Fermented lime	3
Fermented banana(variety Cavendish)	4
Fermented Banana(variety Embul)	4
Fermented Fish	1
Bee-honey	1
Toddy	2
Pudding	2
Yoghurt	0

Though several yoghurt samples from different brand names were used in this research study for the isolation of yeast, all the attempts became unsuccessful (Table 1). A scientific study related to the isolation of yeast from yoghurts in Brazil, conducted by Moreira *et al.*, 2001 states that the improper storage conditions lead to higher

contaminations of yoghurt with yeast. Also he provides a clue that a systematic contamination at the source is also a possible reason even though it was not a reason for the observations in that study. Based on this information, it can be resolved, that yeast isolation failed from the selected yoghurt samples due to the absence of the above mentioned contaminations in the production process. Moreover, the addition of chemicals such as H_2O_2 for the raw milk before processing to extend the keeping quality, practiced by bulk yoghurt producers may be a possible reason for this.

The morphological characteristics of the colonies of isolated yeasts included spherical, creamy smooth, flat types, some with pinkish or yellowish colors, and some with wavy edges. It was especially noticed that all the three yeast isolates obtained from fermented lime samples and the two isolates from mango samples were very creamy compared with others and thus they were predicted to possess beneficial characteristics based on the results of previous studies. Literature provides evidence that yeasts are vastly variable in cell shape, size and colony colors and pinkish to yellowish colony colors are observed due to the accumulation of carotenoids in cells (Libkind, 2012). Rij 1984 has stated that the shape of vegetative cells of yeast is worthwhile as a taxonomic criterion since their form is closely connected with the way in which they are made and in many of the situations the cell shape may be very distinctive that it may be used in generic differentiation.

Characterization of Isolated Yeast Strains

All the yeast isolates gave positive results for the catalase test, showing that all of them can produce catalase enzyme in their cells. Catalase is an enzyme produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H_2O_2. Based on this observation, it was verified that all the isolates are either aerobes or facultative aerobes.

Four out of the 24 yeast isolates gave positive results for the urease test, by indicating a color change from orange to pink. It reveals that 11% of this yeast pool produces urease enzyme which split urea to form ammonia. Yeasts are capable to utilize various nitrogen sources. The ability or inability to utilize nitrate nitrogen is considered to be a valuable diagnostic criterion for decisive purposes. Many genera are characterized by their inability to utilize nitrates, e.g. *Saccharomyces, Kluyveromyces, Pichia* and *Debaryomyces* while in other genera e.g. *Hansenula* all species utilize nitrate. And there are some other genera in which both nitrate positive and negative species occur e. g. *Candida* and *Trichosporon*. All yeasts are basically capable to utilize urea in low concentrations, as the only source of nitrogen when adequate amounts of vitamins are provided and when yeasts are in a media having an organic nitrogen source such as peptone, their ability to hydrolyze high concentrations of urea in that media exhibit some variations. Urea is hydrolyzed by some sporogenous and asporogenous species and ascogenous species are deficient in hydrolyzing urea whereas it is especially marked in the genera *Cryptococcus* and *Rhodotorula* (Rij 1984). As literature reveals, urease positive yeast species have been identified as pathogenically active ones such as *Candida albicans* which cause human diseases. Thus, above urease positive four yeasts were predicted to be pathogenically important ones found in this study.

According to Ebabhi *et al* 2013, yeasts gain carbon typically from hexose sugars, such as glucose and fructose or disaccharides like sucrose and maltose while some species can also metabolize pentose sugars like xylose, alcohols and organic acids. The development of yeast in a carbohydrate source indicates that it can either ferment or utilize it by respiration. These two terms are somewhat confusing since actual assimilation by the cell takes place both during the fermentative and oxidative dissimilatory processes. Literature reveals that assimilation tests are more sensitive than fermentation tests related with discovering the occurrence of enzyme systems since they do not solely depend on the utilization of fermentable sugars and compromise the use of several carbon compounds (Rij, 1984). In this study, Y71 and Y72 were the two isolates that couldn't assimilate D-sucrose suggesting the possibility to be either a *Candida spp* or *Geotrichum capitatum* as observed by previous studies (Obasi *et al*., 2014). They were obtained from fermented mango samples. Only Y57 and Y66 have indicated inability to assimilate D-raffinose. In a study by Obasi *et al.* (2014), *Candida spp, Geotrichum spp, Rhodotorula* and *Kodamaea spp* have exhibited inability to assimilate D-raffinose. Glycerol was not assimilated only by Y74 isolate. According to the studies by Obasi *et al*., 2014, some *Candida* species and *Geotrichum* species were unable to assimilate glycerol. Ability of all the other isolates to assimilate glycerol indicates that these strains possess the glycerol kinase gene (GuT1) and a gene for mitochondrial glycerol 3-phosphate dehydrogenase (GuT2) responsible for glycerol assimilation during fermentative growth. (Obasi *et al*., 2014). D-maltose couldn't be assimilated only by Y63, Y64 and Y74 yeast isolates. In the research study by Gana *et al.* (2014), *Pseudozyma* species were for not being able to assimilate D-maltose. "The ability of the isolates to also ferment maltose shows that they possess uptake mechanism that involves two systems; an energy-dependent maltose permease (ATP to ADP) which transports the maltose intact across the cellular membrane and a maltase (alpha- glucosidase) which hydrolyses maltose internally to yield two glucose units"(Obasi *et al*., 2014).

Eight isolates (Y55, Y56, Y58, Y59, Y63, Y64, Y65 and Y70) were unable to indicate a color change in the medium indicating inability to ferment sucrose. In the research study conducted by Gana *et al*, (2014), all the isolates of *Pseudozyma* species were unable to ferment sucrose, and thus based on this information here these eight isolates can be suggested as belonging to the genera *Pseudozyma.* All the isolates except Y068, Y069, Y073 and Y075 gave positive results for the lactose fermentation test converting the green color of the YPD broth into yellow color, indicating the development of acidity in the media. But only some isolates were able to produce gas during the test exhibiting that they are highly effective in lactose fermentation. Those were the isolates obtained from grapes, banana, mango and honey. The enzyme lactase, β−galactosidase which hydrolyze lactose into glucose and galactose, is produced by many microorganisms that utilize lactose as an energy source. *Kluyveromyces* species exhibit higher activity of this enzyme than other species (Moeini *et al*., 2004). Literature reveals that *S. cerevisiae* is unable to grow and ferment in lactose medium when it is provided as the sole carbon source as it doesn't possess a lactose metabolizing system. ((Moeini *et al*., 2004; Domingues *et al*., 2010). According to Domingues *et al.* (2010), *S. cerevisiae* has majorly adapted to utilize glucose whereas *K. lactis* has adapted to lactose. Therefore, these two yeasts possess different modes of regulation which are responsible for their overall response for carbon sources and this may be the cause for major physiological differences they exhibit. Some *Debaryomyces* species, *Candida* species, *Schizosaccharomyces pombe* and *Mrakia frigida* also provide negative results for the lactose fermentation test (Moeini *et al*., 2004). In the studied yeast pool, 17%, which were lactose non-fermentative, can be predicted to be among these yeast species. In the tested yeast pool, 33%of isolates were identified to be the most effective lactose fermenters as they produced both acid and gas in the experiment. These yeasts were chosen as the best viable candidates for industrial applications related with lactose fermentation (Figure 2).

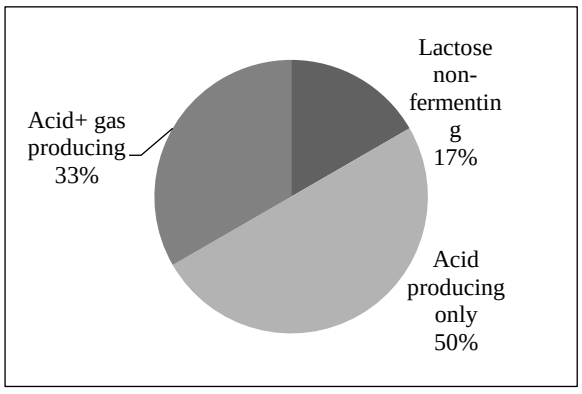

Figure 2 : Effectiveness of the lactose fermentation test

Whey, which is considered as the major waste material in dairy industry can be used as a raw material for the production of ethanol with the use of lactose fermenting yeasts. Thereby, the waste material which causes lot of environmental problems due to its disposal can be converted in to a resource that generates economic benefits. Also ethanol produced from whey through lactose fermentation can be used in products such as potable spirits printing inks and white vinegar. Lactose fermenting yeasts can be incorporated in dairy products for lactose intolerant people.

Based on the above characterization tests and colony morphologies, it was summarized that the tested yeast pool was composed with species belong to genera of *Pseudozyma*, *Cryptococcus*, *Candida*, *Rhodotorula*, *Kluyveromyces*, *Saccaromyces* and *Debaryomyces.* Molecular studies revealed that Y071, isolated from mango to be *Starmerella bombicola* which is an industrially important species due to its ability of producing large amounts (400 g/L) of sophorolipids which are carbohydrate-based, amphiphilic biosurfactants. Due to the biodegradability, low eco-toxicity and the production on renewable-resource substrates, bio-surfactants are beneficial over the chemically synthesized counterparts.

Screening for Industrial Applications

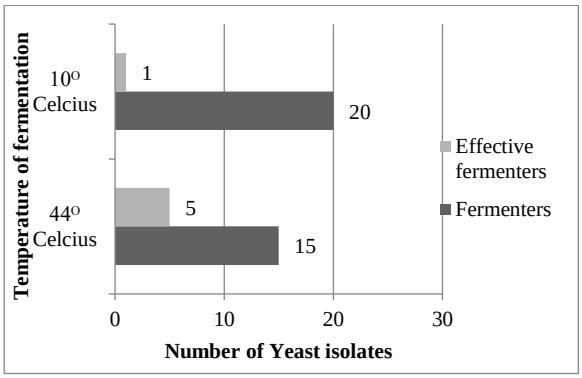

Figure 3 : Effectiveness of the fermentation process under high and low temperatures.

*Isolates which result only in color change are labelled as 'fermenters'. Isolates which result in both gas assembly and color change are labelled as 'Effective fermenters'.

Except four isolates, all others indicated the ability to ferment under low temperature conditions, at 10_oC as provided here (Figure 3). However, Y69 isolated from banana was the most effective at this temperature because gas assembly was also indicated in addition to the color change in the medium. Some products such as cheese and white wine prefer to conduct fermentation under low temperature conditions to achieve the best product properties. Y069 isolate can be suggested as the best viable candidate for such applications. Same observation was recorded from Y55, Y57, Y58, Y59 and Y70 isolates under high

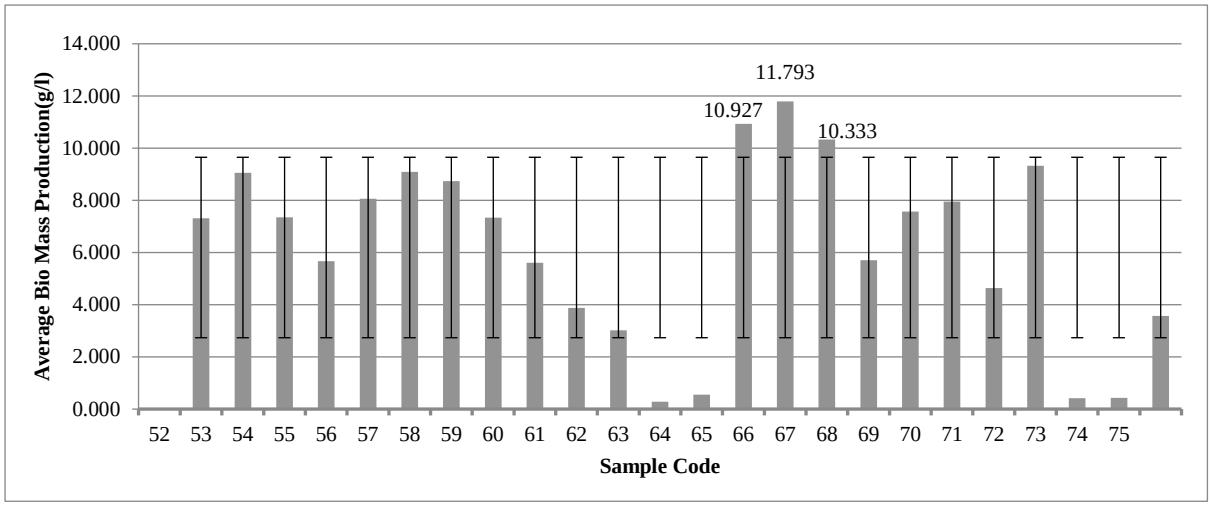

Figure 4 : Average biomass production of the yeast isolates

temperature condition.These thermo tolerant yeast isolates were obtained from fermented banana and grapes samples. Thermo tolerant yeasts can be cultured under conditions where other micro-organisms cannot grow, thus reduces the risk of contamination. The enzymes produced by such strains are also probably thermo tolerant and they are more beneficial due to their eukaryotic nature over the enzymes from bacteria (Takashima *et al*., 2009). Moreover, in tropical and subtropical environments, maintenance of low temperature in fermenters requires cooling systems which generates high production costs especially in sugarcane based fermentations. Thus, use of thermo tolerant yeasts is a good solution to overcome this problem. Based on this study, the five isolates identified as thermo tolerant can be predicted as potential candidates for such applications.

All the yeast isolates indicated color change from green to yellow suggesting that sugarcane juice is a suitable media for the cultivation of them. Sugarcane juice can be used to replace expensive media used under laboratory conditions. But as sugar refining and molasses based distilleries are the major industries in Sri Lanka, using molasses which is produced as co-products of sugar production, to generate ethyl-alcohol is much more economically viable (Chandrasena *et al*., 2006). Thus, screening of yeast isolates that are capable to grow in sugarcane juice must be further screened for their ability to grow in molasses to make it more economically viable in industrial applications.

Average biomass production among yeast isolates when introduced to whey media, exhibited a much variation within the range of 0.280 to 11.793 g/L (Figure 4). Calculated mean value of the data series was 6.180g/L. Based on the results, banana and pudding were the food sources from which the best three biomass producers were obtained. Average bio mass production was highest in Y66 yeast isolate obtained from a pudding sample and it was 11.7933 g/L in amount (Figure 4). A similar bio mass production has been reported in the research study conducted by Moeini *et al*., 2004 and the respected yeast was *K. lactis*. In addition to that, this literature reveals that the bio mass production can be raised further more by using mixed cultures of yeast species such as *K. lactis, K.marxianus* with *S. cerevisiae* and by the supplementation of the medium with a nitrogen source like ammonium sulphate.

Yeast isolates that have exhibited satisfactory level of biomass production have the potential to be utilized in food and fodder yeast production in a successful way. The SCP production using abundant raw-materials is one of the highly demanded applications of yeast micro flora in modern world. There are several benefits of producing yeast based SCPs using whey. The first thing is, whey being the major by-product in dairy manufacturing firms, it causes huge disposal problems due to the high organic matter content. Secondly, whey which contains proteins is not successfully utilized in producing another important product in Sri Lanka. In addition to that, SCPs have a huge demand in current food industries as it has been identified as a good solution to fulfill the dietary protein requirement in increasing populations, especially for vegetarians. Also, SCPs can be used to feed farm animals as a rich source of protein.

In 50% glucose media, only Y066 isolate indicated a growth negative response and it was only a 4% from the total yeast pool. Therefore, 96% of the yeast pool was predicted to thrive well under high osmotic pressure conditions. Thus, they were recognized as suitable yeasts to use in high concentrated food products. Ability to grow at 50% glucose has also been tested in some previous studies as a physiological test to identify yeast strains. According to the research by Gana *et al.* (2014),

Pseudozyma species and *Cryptococcus aerius* have exhibited inability to grow under these conditions.

CONCLUSIONS

There are several foods (fruits and vegetables, fish, bee-honey, toddy and pudding), which are highly locally available could be considered potential sources for the isolation of a variety of yeasts. The isolated pool of yeast was composed of with creamy, circular or irregular, flat, entire, undulate and curled colonies while some of them were pigmented with yellow or pink color compounds. The characterization tests and colony morphology revealed that this pool of yeast belong to genera of *Pseudozyma, Cryptococcus, Candida*, *Rhodotorula*, *Kluyveromyces*, *Saccaromyces* and *Debaryomyces.* Different strains of yeast isolates were identified as viable candidates for utilizing in industrial applications; SCP production from whey, bio ethanol production from lactose containing media, i.e. whey, manufacturing dairy products for lactose intolerant people, fermentations under low or high temperature conditions and production of fermented foods with high concentrations of sugar. Sugarcane juice was recognized as an inexpensive media for industrial cultivation of all the isolated yeasts..

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