Chapter 12
Yeast Isolation Methods from Specialized Habitats

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Contents

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12.1 Introduction

Yeasts are a unicellular heterogeneous group of fungi which were first observed under a microscope by Antonie Van Leeuwenhoek in 1680 and successfully isolated for the first time by Louis Pasteur in the late 1860s. Since 1865, yeast research has undergone significant progress with respect to their distribution, isolation and characterization. Currently, more than 1500 yeast species have been reported from all over the world, which comprise of only 1% of the actual diversity while the remaining still needs to be explored [18]. Yeasts are ubiquitous in nature and have been isolated from aquatic, atmospheric and terrestrial habitats. While some species can be found in great numbers in different habitats, others may be restricted geographically to only a few specialized habitats; suggesting that the overall yeast distribution is not uniform.

It would seem that the distribution of yeasts would not be affected by geographical barriers since they can be dispersed by air currents [34], organismal vectors, plant material as epiphytes or endophytes. But actually, distinct yeast species occur in different regions and seasons. Studies on yeasts associated with beetles, drosophilids, bees, and ephemeral flowers disprove the fact that yeasts are ubiquitous [13, 19, 34]. The yeast diversity of common habitats like soil, plants and other terrestrial habitats has been thoroughly explored since the isolation techniques are easily available and well described [29, 34]. In contrast, the isolation techniques for the less exploited niches (Fig. 12.1) like rumen, insect gut, hot spring, rotten wood, flowers, nectar and other anthropogenic habitats like compost, molasses, distillery wastes etc. are rather cumbersome and not elaborately outlined, since the procedures vary greatly depending on the yeast density, the volume and shape of the source [25]. The correlation between yeasts and the habitats that they occupy is determined by the overall intrinsic factors (chemical, physical and physiological), availability of nutrients, beneficial interactions with other organisms, and the presence of competitors.

In the past decade, several novel yeasts have been isolated from natural habitats which are not fully exploited; one such niche is insect gut [1, 36, 37]. The insect gut has become increasingly recognized as an important source for the isolation of new ascomycetous and basidiomycetous yeast taxa. The major purpose for the yeast-insect association is that the yeasts provide essential amino acids, vitamins, sterols and allelochemicals to attract the insect dispersers for targeted dispersal to a fresh environment [4]. Such associations are believed to have promoted fungal diversity and the expansion of insects into nutrient-poor substrates [37]. Several novel yeasts have been isolated from the gut of beetles. Likewise, the gut of termites has also been explored for novel yeasts, which produce valuable enzymes and can degrade xylan and/or ferment xylose [1] to ethanol. These properties can be exploited in industries for the sustainable production of ethanol or valuable enzymes. The gut of termites and other wood-feeding insects can be considered as natural mini bioreactors with the understudied microbiome, therefore, we have provided a detailed protocol for isolation of such industrially important yeasts from the termite gut.

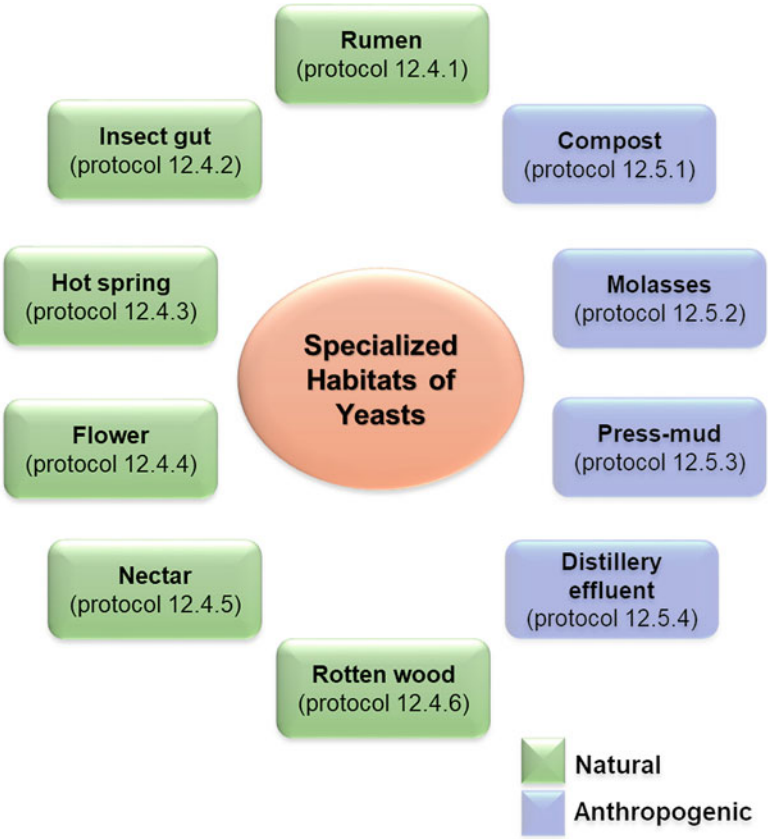

Fig. 12.1 Specialized habitats for isolation of yeasts

In a similar manner, yeasts play a mutualistic role in the rumen of cattle and other ruminants. The rumen is an anaerobic, cellulose and hemicellulose-rich habitat, which harbours many microbes including bacteria, protozoa, filamentous fungi and yeasts [10]. Yeasts isolated from rumen digesta can grow at elevated temperatures since the temperature of rumen itself is 39.6 ± 1 °C. There are few reports of yeasts isolated from the rumen of sheep, bovine, cattle and musk oxen [8, 21, 22]. The exact role of yeasts in the rumen is unclear but it is proposed that they act as probiotics to prevent various diseases in ruminants and stimulate the growth and activity of various fibrolytic ruminal bacteria by providing proteins, vitamins and other growth factors [24, 33]. Since the rumen is also a less explored niche, a protocol for rumen yeast isolation is given in this chapter.

There are some 'wood-feeding yeasts' found on rotting wood, which is a nutrient-rich substrate for yeast populations. This niche has recently received attention after isolation of xylose-fermenting yeasts from rotting wood in Brazilian forests and a novel yeast capable of arabitol production was reported [6, 17]. The hemicellulosic

fraction of wood is broken down to simpler monomers by the yeasts' enzymes. The hydrolytic abilities of the enzymes secreted by these yeasts are of great significance to biotechnological industries, therefore. a brief protocol for yeast isolation from rotting wood has also been given.

Another interesting niche for obtaining novel or rare yeasts is the flower or floral nectar. There are many publications on the properties and characteristics of nectar, but very little is known regarding its role as a natural habitat for micro-organisms, especially yeasts. The presence of yeasts in the nectar of flowers is well known but very little effort has been taken for the isolation of yeasts and studying their role in compositional changes of nectar fluid [16, 43]. It is possible that the yeasts inhabiting nectars of angiosperms play a significant role as intermediate agents in plant-pollinator signalling by the production of volatile compounds by fermentation of available sugars [16, 43]. It is suggested that some osmophilic species can be found in nectar since it contains high levels of sugars, and this trait can be beneficial from a biotechnological point of view.

Some extreme environments have been established as niches for various species of yeasts capable of enduring harsh conditions [34]. One such naturally occurring habitat is the hot spring which harbours thermotolerant and thermophilic yeasts. Novel thermotolerant yeasts have been reported from hot springs [38] which can be used for high-temperature fermentations to produce ethanol. Enzymes like β-galactosidase, cellulase and lipases produced by thermotolerant/thermophilic yeasts are expected to have thermal tolerance, which is one of the most desirable properties of an enzyme to be used for industrial applications [12]. A detailed protocol for the isolation of yeasts from hot springs has been provided in this chapter.

Besides the naturally existing habitats, there are a number of anthropogenic sources from which yeasts can be recovered. Urbanization and human activities have created multiple ecological or functional niches for specific yeast populations. One such artificial habitat is compost. Composting is an aerobic process which involves the microbial conversion of complex organic compounds into their simpler forms [31]. As a habitat rich in carbohydrates and phenolics, composts harbour a variety of microbes including yeasts that have the ability to utilize C5 and C6 sugars and are tolerant to high temperatures and low pH [9, 14]. In the past decade, most research on composts has been focused on their ecological and functional biodiversity. Very few studies have been undertaken to explore yeast diversity from composts despite being one of the rarest and unexplored habitats [9]. Yeasts isolated from composts might be possible candidates for fermentation in biofuel or bioethanol industries.

The yeast biota of carbon-rich substrates like residual juice, molasses and press mud from sugar cane/beet in sugar industries has not been delved into completely. Press mud is also a type of waste which is rich in lignocellulosic material, obtained after ethanol production from molasses and is usually burnt or discarded by most distilleries. Few yeasts of the genera *Saccharomyces*, *Schizosaccharomyces* and *Torulaspora* have been reported from this niche [5, 39, 40]. Molasses from sugar cane and agave are used as the starting material for the production of ethanol or other

distilled spirits [11, 20] and the waste generated (residual molasses and press mud) can possibly harbour high sugar-tolerant or osmophilic microbes, especially yeasts [15, 26, 40]. Isolation of such indigenous yeasts and their employment in fermentation industries can increase the efficiency of ethanol production from molasses [7]. Protocols for yeast isolation from press mud and molasses have been provided.

Much like molasses, distillery waste also constitutes a very rich ecosystem in which varied yeast species can flourish and carry out fermentation spontaneously. The yeast community thrives on residual sugars and other by-products generated after the distillation process to produce ethanol from grape pomace. Very few surveys have been carried out to study the yeast diversity from distillery waste [41, 42]. More recently, distillery waste has also been found to be a potential reserve for novel thermotolerant yeasts [3]. Such yeasts once successfully isolated and characterised can be of interest in the field of biotechnology for efficient production of ethanol from lignocellulosic biomass [27].

The above mentioned natural or anthropogenic ecosystems are all reservoirs of rare and novel yeasts that can significantly contribute to the sustainable advancement of biotechnological research across the globe. Yeast diversity of such specialised habitats has not been completely exploited as a consequence of insufficient knowledge or unavailability of detailed step-by-step isolation protocols. The current chapter presents a comprehensive compilation of available methods that have been suitably modified to successfully isolate yeasts from specialized and less explored habitats like rumen, rotting wood, insect gut, flower/floral nectar, hot spring, compost, molasses/press mud and distillery waste (Fig. 12.1). We aim to provide a complete set of methods as a reference for researchers who are interested in exploring yeast biota from rare ecosystems.

12.2 Common Materials

1. 0.9% saline solution
2. Autoclave
3. Chloramphenicol (HiMedia)
4. Cover-slips
5. Cryo-Vials (2 ml)
6. Deep freezer (−20 °C & −80 °C)
7. Differential Interference Contrast (DIC) Microscope
8. Distilled water
9. Erlenmeyer flasks (250 ml).
10. Ethanol (70%)
11. Falcon tubes (50 ml)
12. Glass slides
13. Glass spreader
14. Glycerol
15. Hand gloves

16. Incubator
17. Laminar air flow cabinet
18. Measuring cylinders (500 ml
19. Micropipettes (P20, P200 & P1000)
20. Milli-Q water
21. Penicillin (HiMedia)
22. Petri-plates (90 mm)
23. pH meter,
24. Plastic bags (6″ × 10″ or 10″ × 16″ size)
25. Plastic containers (500 and 1000 ml)
26. Refrigerated centrifuge
27. Refrigerator (4 °C)
28. Screw-cap glass bottles (500 ml & 1000 ml)
29. Shaker Incubator
30. Streptomycin (HiMedia)
31. Weighing balance
32. Yeast Extract Peptone Dextrose (YEPD) agar plates, (10 g l^{-1} yeast extract, 20 g l^{-1} mycological peptone, 20 g l^{-1} dextrose, 20 g l^{-1} agar; pH 5.0)

12.3 Methods

12.4 Yeast Isolation Protocols from Natural Habitats

12.4.1 Rumen Fluid/Digesta

12.4.1.1 Materials

(a) Common materials
(b) Specific materials:

(i) Whirl-pak® bags
(ii) BagMixer® CC
(iii) Vortex
(iv) Muslin cloth
(v) Manifold machine (anaerobic media preparation)
(vi) Nitrogen cylinder
(vii) Carbon dioxide cylinder
(viii) Serum bottles (120 ml)
(ix) Resazurin (0.1%)
(x) L-Cysteine-hydrochloride (L-Cys-HCl)
(xi) Dispenser (5–50 ml)
(xii) Sealer and de-sealer machines

(xiii) Sterile syringes (1 ml and 10 ml)
(xiv) Syringe needles (gauge 16)
(xv) Potato Dextrose (PD) agar (200 g l^{-1} potato infusion, 20 g l^{-1} dextrose, 25 g l^{-1} agar; pH 5.0±0.5)

12.4.1.2 Protocol

1. Collect approximately 100 g of rumen digesta from buffalo/goat/sheep in sterile whirl-pak® bags (triplicates) from a slaughterhouse or any other source and transfer them to the laboratory immediately.
2. Store all rumen samples at 39.6 ± 1 °C in an incubator until further processing. (Note: It is recommended to process the sample as soon as possible to obtain maximum diversity of yeasts. Samples can be stored at 4 °C temporarily).
3. Pool the samples and equilibrate with CO_2 gas using a manifold machine. Take 10 g of this rumen digesta and suspend it into 100 ml of 0.9% saline solution.
4. Homogenize the above solution using BagMixer® CC for 30 seconds or filter through a muslin cloth to obtain a uniform suspension of rumen digesta before it is used for yeast enrichment and isolation processes.
5. Aerobic isolation on solid media:
   (a) Spread 100 µl of appropriately diluted suspension (10^{-3}, 10^{-4} and 10^{-5}) on YEPD and PD agar plates containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
   (b) Incubate the plates at 39.6 ± 1 °C to obtain thermotolerant yeasts, and 30 ± 1 °C for 48–72 h to obtain the complete diversity of yeasts.
6. Aerobic isolation in liquid media (enrichment):
   (a) Inoculate 10 ml of homogenized rumen suspension into 100 ml of YEPD and Potato Dextrose broth containing antibiotics.
   (b) Incubate the flasks at 39.6 °C and 30 °C for 12–24 h at 150 rpm to enrich the indigenous yeast flora.
   (c) After observing considerable growth, spread 100 µl of the above culture broth on YEPD plates and incubate at 39.6 ± 1 °C and 30 ± 1 °C for 24–72 h to obtain yeast colonies.
7. Anaerobic isolation on solid media: serum roll bottle method [23].
   (a) Add 0.5 ml of appropriately diluted rumen suspension (usually 10^{-3} or 10^{-4}) in 10 ml YEPD and PDA media prepared anaerobically in 120 ml glass serum bottles containing 0.1 ml of resazurin (0.1%), 1 g l^{-1} of L-Cys-HCl and antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
   (b) Incubate all bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 24–72 h and inspect regularly for the development of yeast colonies.

(c) Select different yeast colonies after observation of morphology under a DIC microscope and pick them anaerobically in the presence of N_2 gas.
(d) Subculture these colonies on aerobic (YEPD plate) and anaerobic media (serum roll bottles).

8. Anaerobic isolation on liquid media (enrichment):

(a) Inoculate 3 ml of homogenized rumen suspension in a 120 ml glass serum bottle containing 30 ml of YEPD and PD broth with 0.1 ml resazurin (0.1%), 1 g l^{-1} L-Cys-HCl and antibiotics as mentioned above.
(b) Incubate all serum bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 12–24 h at 150 rpm or until considerable growth has been obtained.
(c) Inoculate 100 µl of the above culture broth into serum bottles containing YEPD media with antibiotics.
(d) Incubate all serum bottles at 39.6 ± 1 °C and 30 ± 1 °C separately for 24–72 h.
(e) Select morphologically distinct yeasts by observing under microscope and subculture on aerobic (YEPD plate) and anaerobic media (roll bottles).

9. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.2 Insect Gut (Termite/Beetle)

12.4.2.1 Materials

(a) Common materials
(b) Special materials:

(i) Forceps
(ii) Xylose
(iii) Stereomicroscope
(iv) Ethanol (90%)
(v) Sterile syringe (5 ml)
(vi) Yeast Nitrogen Base (YNB) + 1% xylose (pH 5.0)

12.4.2.2 Protocol

([35] with few modifications)

1. Collect 15–30 live termites/beetles from rotting wood logs, soil, bark of trees and transfer them to the laboratory at ambient conditions.
(Note: Dead termites/beetles are difficult to dissect and affect the gut micro-flora, therefore, it is important to keep the insects alive until dissection)

2. Surface-disinfect the insect body using 95% ethanol for 2 min and then rinse with sterile distilled water.
3. Dissect and remove the gut from the insect under a stereomicroscope using dissection needles/forceps and transfer the gut into a separate tube for crushing in 0.9% sterile saline solution.
4. Pass the gut suspension through a 2 or 5 ml syringe twice to macerate the gut contents.
5. Direct isolation on solid media:
   (a) Spread 100 µl of the crushed suspension onto YEPD and YNB + xylose plate containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (Note: Do not autoclave YNB+ xylose medium; sterilize by filtration)
   (b) Incubate all plates at 25–27 °C for 24–96 h and observe frequently for growth of yeast colonies.
6. Enrichment in liquid media:
   (a) Inoculate 1 ml of gut suspension into 10 ml of YEPD and YNB + xylose media in 50 ml flasks, each containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all flasks at 25–30 °C for 24–48 h at 150 rpm.
   (c) After observing considerable growth, spread 100 µl of the culture broth on YEPD and YNB + xylose plates containing antibiotics and incubate at 25–30 °C for 24–72 h.
7. Select well-separated, morphologically different yeast colonies and streak on agar plates (YEPD and YNB + xylose) to obtain purified cultures.
8. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.3 Hot Spring

12.4.3.1 Materials

(a) Common materials.
(b) Special materials:
   (i) Hand shovel
   (ii) Ice bucket
   (iii) Membrane filter (0.45 µm)
   (iv) PD medium (pH 5.0)

12.4.3.2 Protocol

([2] with few modifications)

1. Collect approximately 1000 ml of water and 500 ml of wet sediments from hot springs in sterile plastic containers.
2. Store the samples at 4 °C while transferring to the laboratory and process immediately.
3. Concentrate the hot spring water sample by passing through a membrane filter and resuspend the membrane filter into 10 ml of 0.9% saline solution to obtain a uniform suspension.
4. Mix wet sediment samples thoroughly before isolation of yeasts.
5. Direct isolation on solid media:
   (a) Spread 100 and 200 µl from the concentrated hot spring water suspension / wet sediments onto YEPD and PD agar plates with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all plates at 30, 35, 40 and 45 °C separately for 24–96 h or until yeast growth is observed.
   (Note: High incubation temperatures are employed for isolation of thermotolerant/thermophilic yeasts)
6. Enrichment in liquid media:
   (a) Inoculate 2 ml of concentrated hot spring water suspension and wet sediment separately into 20 ml of YEPD and PD media with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate all flasks at 30, 35, 40 and 45 °C separately for 12–96 h at 150 rpm.
   (c) Take 5 ml culture broth from the above flask and inoculate into 100 ml YEPD medium with antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (d) Incubate each flask at 30, 35, 40 and 45 °C separately for 12–18 h at 150 rpm.
   (e) After observing considerable growth, spread 100 µl of culture broth on YEPD plates containing antibiotics and incubate at 30, 35, 40 and 45 °C separately for 24–72 h.
7. Examine yeast morphology microscopically; select different yeasts and streak on YEPD/PD agar plates to obtain purified cultures.
8. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.4 Flower

12.4.4.1 Materials

(a) Common materials.
(b) Special materials:

(i) Mortar and pestle
(ii) Muslin cloth
(iii) PD medium (pH 5.0)

12.4.4.2 Protocol

([32] with slight modifications)

1. Collect fresh flowers and transfer them to the laboratory for processing.
2. Rinse the flower with sterile distilled water for 10 min to remove adhered dust particles and other contaminants.
3. Dry the flower and surface-sterilize by transferring it into 70% ethanol for 2 min.
4. Rinse the flower again in sterile distilled water for 5 min and air dry maintaining sterile conditions.
5. Take 1 g of surface-sterilized flower in mortar and pestle and crush thoroughly in 3 ml sterile water.
6. Add 7 ml sterile water to this slurry, mix well and filter through muslin cloth to obtain a uniform suspension.
7. Spread 100 µl of the appropriately diluted (10^{-4} and 10^{-5}) suspension on YEPD and PD agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
8. Incubate the plates at 25 to 30 °C for 24–96 h and observe frequently for yeast colonies.
9. Select well-separated yeast colonies and observe under a microscope for morphological differences; subculture on YEPD/PD agar plates to obtain purified colonies.
10. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.5 Nectar

12.4.5.1 Materials

(a) Common materials.
(b) Special materials:

(i) Eppendorf tube (1.5 ml and 2 ml)
(ii) Sterile syringe (1 ml)
(iii) Yeast Maintenance (YMA) media (3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} mycological peptone, 10 g l^{-1} glucose, 25 g l^{-1} agar; pH 5.5)

12.4.5.2 Protocol

([43] with apt modifications)

1. Collect sufficient amount of nectar (~1 ml) from inflorescences with a sterile syringe into a 2 ml tube, transfer to the laboratory as soon as possible and store at 4 °C until further processing. (Note: The volume of nectar collected will vary from flower to flowerFlowers; some flowers may have as little as 5 µl of nectar fluid)
2. Dilute 100 µl of nectar with 900 µl of sterile distilled water and spread 100 µl of diluted nectar on YMA and YEPD plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol). (Note: In case of overgrowth of yeast colonies, prepare further dilutions to obtain well-separated yeast colonies)
3. Incubate all plates at 25 to 30 °C for 5 days and observe intermittently for yeast colonies.
4. Select well-separated yeast colonies and streak on agar plates (YMA and YEPD) to obtain purified cultures.
5. Microscopically examine yeast cells to determine their morphology and select different yeasts.
6. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.4.6 Rotten Wood

12.4.6.1 Materials

(a) Common materials
(b) Special materials:

(i) Membrane filter (0.45 µm)
(ii) Sterile syringe (50 ml)
(iii) Yeast Nitrogen Base (YNB) + 1% xylose (pH 5.0)
(iv) YMA medium (pH 5.0)

12.4.6.2 Protocol

([6] with modifications)

1. Collect 50 g of rotten wood in a sterile plastic bag, transfer immediately to the laboratory and process the sample for double enrichment. (Note: Xylanolytic and xylose utilizing yeasts can be isolated through double enrichment as they are present in less numbers)
2. Add 1 g of rotten wood separately into 20 ml of YMA, YEPD and YNB + xylose media each containing antibiotics (100 μg ml^{-1} streptomycin, 100 μg ml^{-1} ampicillin and 25 μg ml^{-1}chloramphenicol).
3. Incubate all flasks at 25 °C for 3–10 days at 150 rpm on a reciprocal shaker.
4. Inoculate 5 ml of the above culture broth into 100 ml of YMA, YEPD and YNB + xylose media containing antibiotics.
5. Incubate all flasks at 25 °C for 12–24 h at 150 rpm on a rotary shaker.
6. Spread 100 μl of culture broth on YMA, YEPD and YNB + xylose plates containing antibiotics.
7. Incubate all the plates at 25 °C for 5 days or until observation of yeast colonies.
8. Select well-separated yeast colonies and streak them on agar plates (YEPD and YNB + xylose) to obtain purified cultures.
9. Select morphologically different yeasts by microscopic observation and streak on YEPD/YNB + xylose agar plates.
10. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) for long-term maintenance.

12.5 Yeast Isolation Protocols from Anthropogenic Habitats

12.5.1 Compost

12.5.1.1 Materials

(a) Common materials
(b) Special materials:

(i) Hand shovel
(ii) Acidified YEPD medium (pH 3.5)
(iii) YEPD 5 (5% dextrose) medium (pH 3.5)
(iv) YEPD 10 (10% dextrose) medium (pH 3.5)
(v) Glass wool
(vi) Glass funnel

12.5.1.2 Protocol

([9] with modifications)

1. Collect approximately 100 g of compost from composting heaps (at least 90 days old) after excavating at a depth of 1 m from the surface and transfer immediately to the laboratory under aseptic conditions.
   (Note: Compost samples can also be stored at 4 °C until further processing, but not for more than 2 weeks)
2. Inoculate 10 g of compost into 100 ml of 0.9% saline solution, to obtain a uniform suspension prior to enrichment and isolation experiments.
   [Note: In order to avoid mould growth, enrich the samples in an acidified liquid broth (pH 3.5) using sulphuric acid, as recommended by [28]. Filter the enriched culture broth with sterile glass wool and use this filtrate for further isolation experiments]
3. Direct isolation on solid media:
   (a) Spread 100 µl of appropriately diluted suspension on acidified YEPD, YEPD 5 and YEPD 10 agar plates containing antibiotics (200 µg ml^{-1}streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
      [Note: Acid hydrolysis of agar at low pH while autoclaving can be avoided by adding the acid to sterilized molten agar (45–50 °C) and mixing gently to avoid air bubbles]
   (b) Incubate the plates at 30, 35, 40 and 45 °C separately for 24–96 h to obtain yeast colonies.
      (Note: High incubation temperatures are employed for isolation of thermotolerant/thermophilic yeasts)
4. Enrichment in liquid media:
   (a) Inoculate 10 ml of suspension into 100 ml of acidified YEPD, YEPD 5 and YPD 10 media with antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate each flask at 30, 35, 40 and 45 °C separately for 12–24 h at 150 rpm.
   (c) After considerable growth has been observed, take 5 ml culture broth from the above flasks and inoculate into 100 ml YEPD, YEPD 5 and YPD 10 media with antibiotics.
   (d) Incubate all flasks at 30, 35, 40 and 45 °C separately for 12–18 h at 150 rpm.
   (e) After observing considerable growth in the flasks, spread 100 µl of culture broth on YEPD and YEPD 10 agar plates containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol).
      (Note: Dilute the culture broth before inoculation in case there is excessive yeast growth)
5. Select well-separated yeast colonies and subculture on YEPD media plates to obtain pure yeast cultures.

6. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.2 Molasses

12.5.2.1 Materials

(a) Common materials
(b) Special materials:

(i) YMA medium (pH 5.5)
(ii) Malt Extract Peptone Dextrose (MEA) agar plates, (20 g l^{-1} malt extract, 6 g l^{-1} mycological peptone, 20 g l^{-1} dextrose, 20 g l^{-1} agar; pH 5.0)
(iii) Sugarcane Blackstrap Molasses (SCBM) medium (2.6% molasses, 0.3% yeast extract, 0.2% KH_2PO_4, 0.1% $(NH4)_2SO_4$, 0.1% $MgSO_4.7H_2O$, 2% agar; pH 5.0)

12.5.2.2 Protocol

([15] with appropriate modifications)

1. Collect 100 ml of molasses from sugar industries in 500 ml plastic containers in triplicates; transfer to the laboratory immediately and store at 4 °C until further processing.
2. Dilute the molasses sample ten times with 0.9% saline solution prior to enrichment and isolation experiments.
3. Direct isolation on solid media:

   (a) Spread 100 µl of the appropriately diluted suspension on YMA, YEPD and SCBMM agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol). (Note: Dilute further if there is excessive yeast growth, e.g. 10−3 or 10−4)
   (b) Incubate the plates at 30 °C for 24–96 h to obtain yeast colonies.

4. Enrichment in liquid media:

   (a) Inoculate 10 ml of appropriately diluted molasses suspension into 100 ml of YMA, YEPD and SCBMM broth, each containing antibiotics.
   (b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
   (c) After observing considerable growth, spread 100 µl of culture broth on YMA, YEPD and SCBMM plates and incubate at 30 °C for 24–72 h to obtain yeast colonies.
   (Note: Dilute the culture broth suitably before plating in case of over-growth of yeasts)

5. Select well-separated yeast colonies; observe under a DIC microscope to select morphologically distinct yeasts and streak on YMA, YEPD, and SCBMM agar plates to obtain purified cultures.
6. Preserve all yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.3 Press Mud

12.5.3.1 Materials

(a) Common materials
(b) Special materials:

(i) Hand shovel
(ii) YMA medium (pH 4.0)
(iii) SCBM medium (pH 4.0)

12.5.3.2 Protocol

([30] with suitable modifications)

1. Collect 100 g to 1 kg press mud from sugar-cane composting heaps in sterile plastic containers and transfer to the laboratory immediately or store at 4 °C until further processing.
2. Suspend 100 g of press mud in 1000 ml of 0.9% saline solution to obtain a uniform suspension prior to enrichment and isolation experiments.
3. Direct isolation on solid media:

(a) Spread 100 µl of the appropriately diluted suspension on YMA, YEPD and SCBMM acidified agar plates (pH 3.5) containing antibiotics (200 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
(b) Incubate the plates at 30 °C for 24–96 h to obtain yeast colonies.
(Note: Prepare serial dilutions (e.g. 10^{-4}, 10^{-5}) in case of overgrowth of yeasts and repeat direct isolation)

4. Enrichment in liquid media:

(a) Inoculate 10 ml of suspension into 100 ml of YMA, YEPD and SCBMM media supplemented with antibiotics.
(b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
(c) After considerable growth has been observed, spread 100 µl of the above culture broth on YMA, YEPD and SCBMM plates and incubate at 30 °C for 24–72 h to obtain yeast colonies.

7. Examine well-separated yeasts under the microscope and subculture them on agar plates (YMA, YEPD, and SCBMM) to obtain purified cultures.
8. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

12.5.4 Distillery Effluent/Spent Wash

12.5.4.1 Materials

(a) Common materials
(b) Special materials:

(i) YMA medium (pH 4.5)
(ii) SCBM medium (pH 4.5)

12.5.4.2 Protocol

[3]

1. Collect approximately 500 ml of effluent from distillery units associated with sugar industries and transfer it to the laboratory immediately under aseptic conditions.
2. Dilute the distillery effluent ten times with 0.9% saline solution prior to enrichment and isolation experiments.
3. Direct isolation on solid media:
   (a) Spread 100 µl of the diluted suspension on YMA and YEPD agar plates containing antibiotics (100 µg ml^{-1} streptomycin, 100 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate the plates at 30 °C for 24–96 h or until observation of yeast colonies. (Note: Prepare serial dilutions (e.g. 10^{-4}, 10^{-5}) in case of overgrowth of yeasts and repeat direct isolation)
4. Enrichment in liquid media:
   (a) Inoculate 10 ml of diluted distillery effluent suspension into 100 ml of YMA and YEPD media containing antibiotics (200 µg ml^{-1} streptomycin, 200 µg ml^{-1} ampicillin and 25 µg ml^{-1}chloramphenicol).
   (b) Incubate the flasks at 30 °C for 12–18 h at 150 rpm.
   (c) Spread 100 µl of the above culture broth on YEPD plates and incubate at 30 °C for 24–72 h.
5. Pick well-separated yeast colonies and subculture on YMA and YEPD media plates to obtain pure yeast cultures.

6. Select morphologically different yeasts and streak them on YMA and YEPD agar plates.
7. Preserve purified yeasts in 15% glycerol at −80 °C and in liquid nitrogen (−196 °C) until further use.

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