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review

Progress in Conventional Methods for Detection and Enumeration of Foodborne Yeasts

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Summary

Genera and numbers of yeasts associated with various types of foods differ according to their ability to tolerate or thrive in environments characterized by wide ranges of temperature, pH, E_h, water activity (a_w) and nutrient content. The performance of a particular medium for detecting different ecological groups of yeasts is also affected by the chemical and physical nature of the food environment. No single medium is satisfactory for detecting, isolating and enumerating all genera of yeasts and in all foods. Antibiotic-supplemented media such as dichloran rose Bengal chloramphenicol agar and tryptone glucose yeast extract chloramphenicol agar are generally superior to acidified potato dextrose agar and other acidified media for enumeration of the vast majority of spoilage yeasts, particularly when cells are in a stressed condition. Dichloran glycerol (18%) agar performs well for enumerating moderately xerotolerant yeasts. Malt extract yeast extract glucose (up to 70%) agar can be used for detecting and enumerating moderate and extreme xerophiles. Lysine agar, Schwarz differential agar and Lin's wild yeast differential agar are used by the brewing industry to differentiate wild yeasts from brewer's strains. Lysine agar is selective for apiculate yeasts and ethanol sulfite yeast extract agar is selective for Saccharomyces. Modified molybdate agar can be used to selectively isolate yeasts in tropical fruits. Preservative-resistant yeasts can be detected on acidified (0.5% acetic acid) tryptone glucose yeast extract agar. The recommended incubation temperature is 25°C, but incubation time between plating and counting colonies ranges from 5 days for determination of general populations of yeasts to 10 days for xerotolerant yeasts. New and improved media for selectively isolating various groups, genera, species and strains of yeasts capable of growing only under specific environmental conditions in specific types of foods and beverages are needed.

Keywords: yeasts detection, yeast enumeration, foodborne yeasts, mycological media

Introduction

Yeasts are distributed widely on the surface of plant materials, both living and dead, and in soil, water and air. Animals may also harbor yeasts, either as a result of contact with their environment or infection. Consequently, unprocessed materials of both plant and animal origin are often contaminated with yeasts at the time they reach the food manufacturer. Processing can either render the finished food product free of yeasts or merely reduce populations. Given enough time, survivors may grow and eventually spoil the product. The detection and enumeration of yeasts in unprocessed and processed foods is an integral part of a good quality management program, and can be used to monitor the effectiveness of sanitation practices at each step during post-harvest and post-slaughter handling, processing, distribution and marketing of foods.

Media for enumerating yeasts in foods should totally suppress the growth of molds and bacteria, be nutritionally adequate to support relatively fastidious yeast species and, in the case of selection for a specific genus, species or group of yeasts, promote the growth of yeasts of relevance to the particular food being examined. Media with such ideal characteristics do not exist. However, several general purpose media have been formulated for enumerating yeasts and molds concurrently, and several others have been concocted to select for specific

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genera or types of yeast. These media enable the food industry to monitor, with some degree of assurance, populations of yeasts in raw ingredients and finished products. Mycological media for isolating, enumerating and identifying yeasts are described in the literature (*1–13*). Proceedings from the first (*7*) and second (*12*) *International Workshops on Standardization of Methods for Mycological Examination of Foods* provide state-of-the-science information on methods for assessing the mycological quality of foods. The following will provide a description of progress made in developing conventional agar media for detecting and enumerating spoilage and fermentative yeasts in food ingredients and processed products.

General Enumeration Media

Several media have been used for the detection, isolation and enumeration of yeasts and molds in foods. Traditionally, acidified potato dextrose agar (pH = 3.5), wort agar (pH = 4.8), malt extract agar (pH = 3.5) and other acidified media have been used because they inhibit colony formation by most bacteria. Media with low pH, however, may yield lower yeast counts than media containing antibiotics (*14–20*). Yeast cells stressed or injured as a result of exposure to physical or chemical assault may not resuscitate at pH = 3.5 (*21*), and lactic acid bacteria may form colonies that interfere with yeast colony development and enumeration. Proteins in food may precipitate in acidified media, thus interfering with enumeration of yeast colonies.

Antibiotic-supplemented media (pH = 5.4–6.8), *e.g.* dichloran rose bengal chloramphenicol agar (*22*) and tryptone glucose yeast extract chloramphenicol agar (*12*), are generally superior to acidified (pH < 4.0) media for isolating and enumerating the highest number of healthy and injured cells of yeasts in foods. Acidified media should not be eliminated from the food mycologist's battery of yeast enumeration media, however, because aciduric strains which may dominate the mycoflora of low pH foods such as fruits and fruit products can usually be recovered on these media. Antibiotics useful in controlling the growth of bacteria in enumeration media have been tabulated (*55,23*.). Bacterial growth can be inhibited by oxytetracycline, chloramphenicol, chlortetracycline, gentamicin and streptomycin at concentrations ranging from 10 to 100 μg/mL. Antibiotics are most effective when they are active against a broad range of bacteria, resistant to inactivation by medium components and food inocula, stable during incubation of the medium and have no effect on the recovery of stressed yeasts (24,25). The use of two antibiotics in selective media may be necessary to control growth of all bacteria. Molds, however, will grow on general antibiotic-supplemented yeast enumeration media, so discretion in counting colonies formed on these media must be exercised when recording yeast counts in food samples.

The choice of antibiotic-supplement medium is dependent to some extent on the nature of the food being examined. Oxytetracycline glucose yeast extract agar (*26*), rose Bengal chlortetracycline agar (*23*), dichloran rose Bengal chloramphenicol agar (*22*), oxytetracycline gentamicin glucose yeast extract agar (*9*), yeast extract glucose chloramphenicol agar (*27*) and tryptone glucose yeast extract chloramphenicol agar (*12*) are used most extensively. An advantage in using chloramphenicol over some antibiotics is that it can be added to media before sterilization. Rose Bengal and dichloran tend to restrict spreading of mold colonies, and thus facilitate the counting of yeast colonies. Tryptone glucose yeast agar without antibiotics is recommended for analyzing foods containing mycoflora consisting mainly of yeasts (*11,28*). There is a need for a general-purpose medium which will support the growth of yeasts while completely inhibiting the growth of molds and bacteria.

Selective Media

Media for enumerating certain species of yeasts in specific types of foods are often desirable in food quality assurance laboratories. A list of 60 media for selecting particular species or genera of fungal plant pathogens and 15 media for human or animal pathogenic fungi has been published (*29*), but only a few media have been developed for food mycologists, and most of these are formulated to select for specific species or genera. Some, however, have been formulated to isolate species of yeasts and even strains within species which are capable of certain metabolic activities. Media suitable for enumerating yeasts indigenous to specific types of foods have been developed (*30*). These media are valuable in assessing the quality of foods and beverages at various stages of processing and during storage. Strategies that may be used to formulate such media have been described by Beech and Davenport (*31*), Phaff *et al.* (*32*) and Miller (*33*), and tabulated by Davenport (*5*) and Jarvis and Williams (*34*). Reviewed here are these and other media formulated to select for specific groups or genera of yeasts commonly associated with foods and beverages.

Acid-resistant yeasts

Growth of most spoilage yeasts can be controlled by the addition of benzoic or sorbic acids to foods. These acids are particularly effective in foods with pH less than ca. 4.5, since inhibitory activity is largely dependent on the undissociated form. *Zygosaccharomyces bailii* and, to a lesser extent, *Z. rouxii* are often capable of growing in foods containing these acids at concentrations as high as 1000–1400 μg/mL(g). Growth can result in film formation on the surface of brines of acetic and lactic acid-preserved pickles and olives. Spoilage of mayonnaise, sauces, fruit juices and wines can also occur. Some strains produce carbon dioxide in quantities sufficient to cause explosive spoilage (*10*).

Zygosaccharomyces species, as well as other acid-resistant yeasts, *e.g.*, *Schizosaccharomyces pombé* and *Pichia membranaefaciens*, that are resistant to high concentrations of benzoic or sorbic acid can be enumerated with some success by plating food samples on malt extract agar acid to which 0.5% acetic acid is added just before pouring (*10,11*). Since only acid-resistant yeasts can grow on this medium, they can be differentiated from other yeasts which may also be present in the food sample (*35*). Other media traditionally used to enumerate acid-resistant yeasts are acidified (0.5% acetic acid) tryptone glucose yeast extract agar (*28*) and *Zygosaccha-*

romyces bailii agar (*36*). Recent studies (*37–40*) have shown that acidified tryptone glucose yeast extract agar performs best in supporting colony formation of acid-resistant yeasts. However, the suitability of acidified media for resuscitating injured yeast cells is less than adequate. There is still a need for an enumeration medium that will exclusively select for acid-resistant yeasts in food products.

Xerotolerant (osmophilic) yeasts

Yeasts capable of growing in foods with reduced water activity (a_w) have been described as osmophilic, osmotophilic, osmotolerant, osmoduric, osmotrophic, xerophilic or xerotolerant. Although the term osmophilic is commonly used, xerotolerant is preferable because yeasts that can grow at a_w = 0.62 to 0.85 generally do not have an absolute requirement for reduced a_w and, in fact, may grow most rapidly at a_w = 0.92 to 0.97. The range of a_w within which a yeast can grow to be classified as xerotolerant is not always clear. The lowest a_w at which most nonxerotolerant yeasts can grow is in the range of 0.85 to 0.92. Whatever the a_w tolerance might be, yeasts that are capable of growing in an environment with reduced a_w can be isolated from foods by plating on media containing substantial amounts of sugar(s) or other polyols or, for halotolerant yeasts, sodium chloride (*2,13,41–44*). The medium chosen for detection or enumeration of xerotolerant yeasts should reflect the content of the food being analyzed. Glucose-, fructose-, sucrose- or glycerol-supplemented media should be used for analysis of high-sugar products, while media containing sodium chloride is generally more suitable for high-salt foods.

None of the media formulated for the purpose of isolating or enumerating xerotolerant fungi is completely selective for yeasts, and only one, dichloran 18% glycerol agar (*45*), is commercially available. Glycerol lowers the a_w of the medium and dichloran restricts spreading of most mold colonies, particularly those of mucoraceous species that otherwise tend to spread over the agar surface and interfere with detecting slower growing xerotolerant molds and yeasts.

Malt extract yeast extract glucose agar containing 20 to 60% glucose was developed by Pitt and Hocking (*10*) for the purpose of isolating and enumerating xerotolerant and xerophilic fungi, including yeasts. Supplementation with 20, 40, 50 or 60% glucose results in a_w values of 0.97, 0.93, 0.89 and 0.85, respectively. The formula selected depends on the type of food under investigation and, the species of xerotolerant yeast suspected to be present in the food.

Glucose citric acid tryptone agar (pH = 4.0) was developed by Ingram (*46*) for enumerating sugar-tolerant yeasts in concentrated orange juice. This medium has some disadvantages in that colonies develop slowly and are nearly transparent. Ingredients must be sterilized separately to avoid hydrolysis and other undesirable chemical reactions, making preparation laborious and time consuming. Scarr's osmophilic wort agar (pH = 4.8) was also developed for enumerating xerotolerant yeasts in high-sugar products (*47*). Colonies of *Zygosaccharomyces* can be counted after 3 or 4 days incubation at 27 °C. Easily countable colonies of less xerotolerant yeasts may require 5 days to develop.

Malt extract yeast extract agar containing 50% sucrose has been used satisfactorily for enumerating xerotolerant yeasts in jams, jellies, fruit concentrates and dried fruits. Potato dextrose agar containing 60% sucrose (*48*) and a yeast extract medium containing 50% glucose (*49*) have been reported to perform well for enumerating xerotolerant yeasts in foods. Media highly suitable for selective enumeration of xerotolerant yeasts in these and other reduced-a_w foods have not been perfected. Such media would be of great value to the food industry.

Alcoholic beverages

Several media have been developed for general quality control programs in the brewing industry. Universal beer agar medium (pH = 6.3) and brewer's tomato juice medium (*50*) can be used to isolate most culture yeasts, non-culture yeasts and Gram-negative bacteria commonly found in breweries. Incubation under anaerobic conditions is necessary for accurate enumeration of *Lactococcus, Leuconostoc* and *Pediococcus*, the most common lactic acid bacteria which cause defects and spoilage of beer. When supplemented with cycloheximide (4 μg/mL), these media differentially suppress the growth of brewer's yeast (*51*). Brewer's yeast and most other *Saccharomyces* species cannot utilize lysine as a sole source of nitrogen, while other yeast genera that occur as brewery contaminants can. Although the absence of yeast growth on lysine agar (*52*) does not necessarily mean an absence of wild yeasts, the medium can nevertheless be used to monitor for the presence of contaminants.

Growth of *Sacch. cerevasiae* is retarded on Schwarz differential medium (*53*), which contains a fuchsin-sulfite mixture as a selective agent; however, this medium supports the growth of wild yeasts. Some strains of *Sacch. carlsbergensis* grow slowly, but colonies are distinguishable from those formed by *Sacch. cerevisiae*. Many wild yeasts can be detected on Lin's wild yeast differential agar (*15,54,55*) on which *Saccharomyces* strains used brewery culture yeasts cannot grow. However, *Torulaspora delbrueckii* and *Kluyveromyces marxianus* cannot be detected using this medium. Both of these yeasts can be detected using lysine agar.

Natural fermentation of grape musts, *i.e.* without the addition of a starter culture, is preferred by some wine producers. In either case, alcohol-tolerant *Sacch. cerevisiae* eventually dominates most fermentation (*56, 57*). Species of *Kloeckera, Hanseniaspora, Candida* and *Pichia* naturally present in grapes musts proliferate during the first 3 or 4 days of fermentation and are thought to contribute significantly to sensory characteristics of wine. Knowledge of populations of wine yeasts, either naturally present or inoculated, as well as apiculate yeasts during various stages of fermentation is therefore desirable, and selective media have been developed for this purpose.

Ethanol sulfite yeast extract agar is selective, but not exclusive for *Saccharomyces* (*58*). The medium can be used to enumerate low populations of *Saccharomyces*; however, other ethanol-tolerant yeasts such as *P. anomala*

may occasionally also form colonies. This medium is especially valuable for enumerating *Saccharomyces* during the early stages of fermentation when apiculate yeasts predominate. Lysine agar (*52,55*) is useful to select for apiculate yeasts during wine fermentation, since lysine cannot be assimilated by *Saccharomyces* species. The medium is therefore useful to the wine industry in monitoring non-*Saccharomyces*, species capable of assimilating lysine. Malt extract agar, a non-selective medium, and ethanol sulfite yeast extract agar and lysine agar were evaluated by Heard and Fleet (*43,59*) for their efficacy in supporting growth of *Saccharomyces* and selecting for non-*Saccharomyces* species, respectively, during fermentation of wines. Malt extract agar provided reliable information on populations of *Saccharomyces* but, because wine yeast predominates as fermentation progresses, accurate measurement of most non-*Saccharomyces* species is not possible. As populations of *Saccharomyces* increase, dilutions necessary to enumerate *Saccharomyces* also increase, thus resulting in the absence of lower populations of other yeasts on malt extract agar plates. Lysine agar was suitable for monitoring growth of *K. apiculata, Saccharomycodes ludwigii* and *Candida stellata*, although the latter yeast formed small colonies on malt extract agar. Ethanol sulfite yeast extract agar had little advantage for enumerating *Sacch. cerevisiae*. Technical difficulties in maintaining an accurate concentration of ethanol in this medium resulted in inconsistencies in suppressing growth of yeasts other than *Saccharomyces*.

Other selective media

Molybdate agar was formulated as a selective and differential medium for isolating yeasts from clinical specimens (*60*) and modified by supplementing with 0.125% calcium propionate and 3% agar for the purpose of selecting for yeasts in tropical fruits (*61*). When compared to malt extract yeast glucose peptone agar, modified molybdate agar was superior for isolating higher populations of yeasts from chikoo, cashew nut, passion fruit, mulberry and guava. Increases of 10- to 100-fold were obtained on the molybdate medium. Recovery of *Saccharomyces, Debaryomyces, Rhodotorula* and *Cryptococcus* species was particularly good.

A selective medium based on yoghurt has been developed for the enumeration of yeasts in foods (*62*). The medium is reported to out-perform antibiotic-supplemented plate count agar and acidified potato dextrose agar in enumerating yeasts in a wide range of food products.

Supplementation of media with sodium or calcium propionate has been reported to aid in the selection of yeasts in mold-contaminated foods (*63,64*). The concentration depends on the pH of the medium, but a range of 0.1 to 0.2% can be effective. Diphenyl is less effective than propionate in inhibiting mold colony development (*65*) but insolubility renders its use inconvenient and its volatility results in loss of fungistatic properties after prolonged incubation.

Simultaneous detection and enumeration of foodborne yeasts possessing specific enzyme-producing capabilities, *e.g.* protease, lipase, pectinase or amylase activity, is occasionally desirable. A procedure to detect proteolytic and lipolytic yeasts without the necessity of picking and analyzing all colonies has been described (*18*). When proteolytic yeasts are to be studied, 0.4% gelation is added to three-quarter-strength antibiotic-supplemented plate count agar before sterilization. Proteolytic yeasts form colonies surrounded by clear zones on this slightly opaque medium. Lipolytic activity can be detected by adding 0.01% calcium chloride and 0.5% Tween® 40 to the basal medium. Lipolytic yeast colonies form a halo of precipitated calcium palmitate and thus can be distinguished from non-lipolytic yeasts. These media performed well for detecting proteolytic and lipolytic yeasts in meats, seafoods, cheeses, orange juice, salad dressing and pizza. Media based on single carbon, nitrogen or vitamin sources which can be used to isolate and identify specific groups of yeasts and yeast species are described by van der Walt (*66*).

Conclusions

While progress has been made in developing agar media for detection and enumeration of yeasts in foods, still improvements need to be made. Development of a medium that supports the growth of yeasts but prevents or greatly inhibits the growth of molds deserves more research attention, as does the development media selective for specific yeast species. Particularly pressing is the need for media selective for yeasts with exceptional resistance to benzoic and sorbic acids or with high tolerance to reduced a_w. The future will bring new methods for rapid detection and identification of foodborne yeasts based on molecular technologies, *e.g.* DNA fingerprinting, polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis and restriction fragment length polymorphism (RFLP) analysis (*67*). However, there will continue to be a need for enumerating foodborne yeasts using conventional media and yet to be developed agar media as a part of comprehensive quality assurance programs in the food industry.

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Napredak u konvencionalnim postupcima otkrivanja i determiniranja kvasaca u namirnicama

Sažetak

Rodovi i brojevi kvasaca, povezani s različitim vrstama hrane, razlikuju se prema njihovoj sposobnosti da opstanu ili uspijevaju u okolišu relativno velikog raspona temperature, pH, E_h, aktivnosti vode (a_w) i količine hranjivih tvari. Na sposobnost određene podloge da otkrije različite ekološke skupine kvasaca utječe kemijska i fizička priroda okoline namirnica. Ne postoji niti jedna pojedinačna podloga za otkrivanje, izolaciju i determinaciju svih rodova kvasaca u svim vrstama hrane. Antibiotikom dopunjene podloge kao dikloran Bengal-ružičasto kloramfenikol agar i tripton glukoza kvaščev ekstrakt kloramfenikol agar općenito su bolje od zakiseljenog dekstroza (od krumpira) agara ili drugih zakiseljenih podloga za determinaciju velike količine kvasaca kvarenja, osobito ako su stanice u stresnim uvjetima. Dikloran glicerol (18%) agar omogućava determinaciju umjereno kserotolerantnih kvasaca. Sladni ekstrakt kvaščev ekstrakt glukoza (do 70%) agar može se koristiti za otkrivanje i determinaciju umjerenih i ekstremnih kserofila. Lizin agar, Schwarzov diferencijalni agar i Lin-ov divlji kvasac diferencijalni agar koriste se u pivovarstvu za razlikovanje divljih kvasaca od pivskih sojeva. Lizin agar je selektivan za apikulatne kvasce, a etanol sulfit kvaščev ekstrakt agar je selektivan za sojeve Saccharomyces. Modificirani molibdat agar može se koristiti za selektivnu izolaciju kvasaca u tropskom voću. Kvasci otporni na konzervanse mogu se otkriti na zakiseljenom (0,5% octene kiseline) tripton glukoza kvaščev ekstrakt agaru. Preporučljiva je inkubacija pri 25 °C, dok vrijeme inkubacije između nasađivanja i brojenja kolonija iznosi od 5 dana za određivanje ukupne količine kvasaca do 10 dana za kserotolerantne kvasce. Potrebne su nove i poboljšane podloge za selektivnu izolaciju raznih skupina, rodova, sojeva i vrsta kvasaca sposobnih da rastu samo pod specifičnim uvjetima okoliša u različitim vrstama hrane i pića.