A Study on Identification of Rhizopus and Bacillus Species Associated with Spoilage of Bread Samples

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Abstract

Bread is regarded as a semi-perishable food and one of the world's staple foods. Bread usually deteriorates as a result of incorrect preservation. The fungus that causes bread to decay has been the subject of experiments by researchers. The degeneration of bread mould is brought on by contamination during post-processing. The microbial bread degradation and the waste problem it causes cause considerable financial losses for both the bakery business and the client. Mycotoxins, which are caused by fungal infection in grains and grain products, are still a significant issue. The creation of clean-label substitutes is important due to the numerous drawbacks of employing traditional chemical preservatives. In this article, we summarize recent research that intends to prolong bread's shelf life by substituting ecologically responsible and consumer-friendly preservation techniques for chemical additions.

Keywords: Bread mold, *Rhizopus sp*, *Aspergillus sp*, *Penicillium sp*, nutritional agar.

1. Introduction

Everyone like eating bread since it is a food product that is widely regarded to be highly handy, whether they are wealthy or poor, living in rural or metropolitan locations. It is one of the most well-liked and widely accepted fundamental meals in existence today and has roots in the Neolithic era. It has a variety of macro and micronutrients that are all essential for preserving human health. The deteriorating of bread and other bakery goods can be caused by physical, chemical, and microbiological factors; the latter is the most problematic, especially when it comes to the growth of bacteria (Bacillus sp.) and mould. Rhizopus is one of the moulds in charge of bread deterioration. (2015) P. Saran Raj and P. Shivasakhthivelan. When stored in a setting with high relative humidity and high relative temperature, the bread moulds. Bread moulds can appear in a variety of colours, including white, golden yellow, and green-grey, depending on the species and degree of sporulation. (2011) Nikolaos Loannis et al. Mould is the fuzzy growth that fungus causes on damp or rotting organic waste. Fungi are found in over a million various animal species. Fungi species are similar to plants but lack chlorophyll. Fungi cannot produce chlorophyll, thus they must get their nutrition from other sources. Fungi cannot produce chlorophyll, thus they must get their nutrition from other sources. Fungi may thrive in damp, dark environments. Numerous mycotoxins have significant antibiotic properties that might lead to cross-resistance in bacteria to some of the most commonly used antibiotics in use today, such penicillin. Mycotoxins are produced as a result of food mould development. Some mycotoxins are exclusively found in mould, whereas others are secreted in food and are the majority. Because mycotoxins may quickly spread in liquid meals and fruits like peaches, pears, and tomatoes, no portion of the food is immune to contamination. Food spoilage is usually caused by moulds, yeasts, and bacteria that are present in the soil, water, and air. Raw fruits, baked products, and many processed meals include enzymes that are susceptible to degradation on a physical, chemical, and microbiological level as well as unfavourable changes in flavours, colours, and textures. Fungus contamination poses the biggest threat to the long-term shelf life of baked products. According to Yehia, Aly El Sheikha (2015), mycotoxins—toxic compounds made by moulds that target a range of foods—are extremely detrimental to both human health and life. Foods that have been baked are regarded as one of the most important items in the food industry. Every day, people consume bread, which is a basic meal in many countries. The production of bread in the European Union (EU) remains essentially stable, with no growth in the majority of western countries. Less than 50 kilogrammes of bread are consumed yearly per person in the UK and Ireland, compared to an average of 80 kg annually by Germans and Austrians.

In contrast to Europe, most emerging economies are experiencing an increase in bread product consumption. Consumer behaviour in the baked goods industry has altered during the last 10 years. Particularly, consumer worries about food safety and additive content have gotten a lot of attention. Additionally, more customers are starting to care about their health. Consequently, there is a substantial market demand for "natural" and "wholesome" meals free of additives and chemically derived preservatives. Therefore, bakers are putting forth more effort to provide "clean label" products that meet their clients' better lifestyles. These goods' food labels use phrases like "natural" and "no preservatives." In reality, increasing the proportion of wholegrain and removing additives/preservatives, which accounted for 21% of new products produced in Europe in 2013/2014, continues to support the creation of new items with a focus on health.

Food with a short processing time, no artificial ingredients, and no chemical preservatives should be of great quality and have a long shelf life. Bacteria, yeast, and mould can all contribute to the deterioration of bread. On the other hand, contamination after baking is often brought on by fungal spores from the bakery environment. The four parameters oxygen, temperature, pH, and water activity (aw) have the most effects on controlling the growth of unwanted fungus in foods. Due to their high moisture content and aw values between 0.94 and 0.97 at a pH of roughly 6, sliced, packed, and wrapped bread are some of the bakery products that are most vulnerable to mould growth. In general, bread contains a lot of moisture. This keeps moisture from escaping from the bread slices during wrapping, preventing fungus from growing in a damp environment. Freshly baked bread without added preservatives has a short shelf life at room temperature when it is wrapped. The waste issue caused by the microbiological degradation of bread continues to be very expensive for both the bakery business and the customer.

According to a 2011 Novozymes survey of over 4000 bread customers in Europe, the most common reason for bread being thrown away was because it had grown mouldy. Mould is responsible for more than 20% of domestic read losses in the UK; this waste, which amounts of 65,600 tonnes of bread slices, costs £72 million per year. Fungi are responsible for the unsightly look of visible mould development in addition to creating mycotoxins and off flavours, which can occur before apparent mould growth. As a result, those who consume deteriorating bread risk serious health risks. Post-baking impurities in bread can be eradicated using physical procedures such as ultraviolet light, infrared radiation, microwave heating, or ultra-high-pressure treatments. However, there haven't been many contemporary studies that have employed these methodologies. As a result, bio preservation, defined as "the extension of shelf life through the use of natural or controlled microbiota and/or their antimicrobial compounds," is becoming a more important topic of study. Such chemicals might alternatively be referred to as "active plant components" or "plant extracts". In addition, current research has focused on strategies to improve protective packaging. This study looks at the possibilities for bio preservation and smart packaging to ensure bread safety, with an emphasis on antifungal compounds produced in situ by lactic acid bacteria (LAB), yeast, and plant-derived chemicals (Axel. c. et al, 2016).

2. Methodology

The research strategy focused on key literature databases such as SCOPUS, PubMed, ResearchGate, and Google Scholar. The keywords mentioned in Table 1 were used to discover articles, beginning with the major keywords and connecting them with the subsidiary keywords using the set operator AND. The references to the paper were also evaluated in order to determine further relevant articles. This review considered investigations on the detection of Rhizopus and Bacillus species related with bread deterioration. This topic does not contain articles on nutritional analysis, bread formulation and innovation, or the functional ingredients of bread. For this article, no research with equivocal results were considered. Finally, with the exception of a few key concepts, only papers focusing on current advancements in this field were accepted in order to investigate this clean-level alternative.

Table 1: A literature search combining these keywords has enhanced us to write this review.

Primary Keywords	Secondary Keywords
Bread mold.	Spoilage of bread.
Fungus incorporating bread spoilage.	Types of bread spoilage.
Conditions responsible for bread spoilage.	Early detection of spoilage.
Pathogenicity of types of bread spoilage.	Mycotoxin production and prevention from spoiled bread.

3. Review of Previous Studies

Bread is the most popular perishable food in the Western world. Unfortunately, bread only remains "fresh" for a short time after it has been removed from the oven. It goes through a lot of changes while being preserved, which causes it to lose its organoleptic freshness. The two primary types of variables influencing how rapidly bread loses freshness during storage are those caused by microbial assault and those caused by a series of subtle chemical or physical changes that lead the crumb to progressively firm up, a process known as "staling" (Pateras, 1999).

Bread has been a staple diet since the Neolithic era. Political power has been used to impose control over the production and distribution of bread for at least the last two thousand years. Wheat flour is the most important ingredient in baking. Soft wheat flour has a low protein concentration and is used to produce biscuits and other pastries, whereas hard wheat flour has a high protein content and is used to manufacture a range of other items. Other bread kinds, such as whole-grain bread, have high fibre concentrations that help the digestive system work properly. Rye has the most nutritional fibre, and as a result, its products have a lower glycemic index than those manufactured from wheat, making them suitable for diabetics (Mondal and Datta, 2008).

Bread is an important part of the Western diet. When bread is exposed to the elements for an extended period of time, it loses its freshness (Pateras, 1999). Making bread necessitates a large amount of wheat, and the composition of the flour is determined by the quality of the grains utilised. The amount of milling has an effect on the chemical makeup of the bread. Bread is now an essential part of the human diet. Whole wheat flour has beneficial elements. It has an acceptable amount of fibre, antioxidants, vitamins, and minerals, according to Kourkouta (2017). Because of its low fat content, high dietary fibre content, and inclusion of essential amino acids, barley seed is nutritionally similar to or more nutrient-dense than other cereals. Barley flour is a healthy food because it includes b-glucans, which have been shown to lower blood cholesterol and glycemic load. Com flour is used in the production of bread, cake, doughnuts, and other pastry goods. It contains a lot of vitamin A and a lot of carotenoids, which function as antioxidants. Furthermore, because these nutrients are concentrated in the fruit's outer layers, whole wheat flour has a higher percentage of them than traditional wheat flour (Kourkouta, 2017).

4. Bread and its Types

White bread

It has a moderate protein level and a high phosphorus, iron, sodium, moisture, and fat content. It has a low calcium content. There are no other differences in its constituents than salt and sugar. You can make white bread with flour, water, salt, and yeast. The bread has a light color and a flavour that is wonderful when first tasted, but it quickly degrades after losing its freshness. White bread's nutritional content is suitable for boosting their energy levels, young people and athletes. It has a deep flavour and is velvety (food and nutrition, 2018).

Fig 2: White bread

White bread has 67 calories, 1 gramme of total fat, 13 grammes of carbs, and 2 grammes of protein.

White bread contains almost the same amount of protein and carbohydrates as whole meal bread, which also contains both soluble and insoluble dietary fiber and a sizable portion of the nutrients found in whole wheat. It is created with unbleached flour that is 78% of the wheat grain's interior if you prefer. You can obtain more fiber from other meals, such wholegrain cereals, in addition to white bread or wholegrain bread.

Whole meal bread is manufactured with at least 90% whole grain flour in New Zealand. To manufacture items containing wheat meal, white flour may be mixed with whole-grain flour. Because of the gluten proteins it contains, it is frequently added to bread that is baked with whole wheat flour to enhance the baking quality. Since there are no food standards governing wheat meal bread, different amounts of whole meal flour may be utilized.

Brown bread

It contains just a tiny bit of bran and germ. White bread is less nutrient-dense than this. It is rich in minerals, vitamin B, and protein. Additionally, it offers vital nutrients like magnesium, potassium, calcium, iron, and sodium. After baking, it has a brownish appearance with a crunchy crust, a delicious aroma, and flexible crumb texture (Barbarisi, 2019).

Brown bread has 92 calories, 2 grammes of total fat, 17 grammes of carbs, and 3 grammes of protein.

Fig 3: Brown bread.

Health Advantages

Energy is obtained from carbohydrates, which make up the majority of the calories in bread. Carbohydrates are the preferred source of energy for our body. When we eat bread, we provide our bodies the fuel they require for our everyday tasks. According to our research, white bread negatively impacted health status whereas whole wheat bread had no obvious influence on diabetes risk variables. Whole wheat bread's efficacy has to be further investigated to see whether it has a good influence on health because it has a low content of soluble fiber (Askar et al. 2013).

Microbiology of bread

One of the significant staple foods in the majority of nations is bread. The two main ingredients are flour and water. There are many different types, sizes, and textures thanks to the blending of various flours and components including salts, fats, yeasts, eggs, sugars, spices, fruits, vegetables, nuts, and seeds. Carbohydrates, proteins, fats, vitamins, fiber, and minerals are all abundant in bread. Bread can be prepared in a variety of ways, consumed as a snack, and even used to other dishes as an ingredient. Bread has gained significance as a staple food around the globe in religious rites, secular cultural life, and linguistics. Problems with physical, chemical, and microbiological deterioration can occur with bread. Bread's primary economic value is mould development, which is a big and expensive problem for bakeries and consumers (Saran raj et al., 2012). The high temperature employed in the baking process will result in the development of spores if the raw materials are contaminated with bacteria that produce rope. Additionally, the moist atmosphere created by the baking process will promote the growth of the bacteria's vegetative cells and spore germination (Yi bar et al., 2012) Since ancient times, microorganisms have been utilized to create bread, cheese, yoghurt, and wine. Today, microorganisms are still used by food producers to create a variety of food products through the fermentation process. In addition to improving food's flavors, texture, and aroma, fermentation also brings about changes that inhibit the growth of unwelcome food microorganisms. The food is safer and has a longer shelf life as a result. These days, fermentations are employed to create an astounding variety of foods and beverages. For thousands of years, people have exploited fungi as food sources and for food preparation. Different fungi have been utilized to complement and flavors dishes in addition to being eaten directly, such as edible fruiting structures like mushrooms. In the fermentation of fruits to make wine, grains to make beer, the making of bread, and flavoring in the form of yeast extract, yeasts are used. Traditional methods for cheese ripening and the creation of enzymes used in the food sector both employ filamentous fungus. To make bread, sugar, flour, and warm water are combined with a yeast called Saccharomyces cerevisiae. The sugar and the other sugars in the flour serve as the yeast's sustenance.

Early detection and differentiation of bread spoilage

Given the push from consumers and lawmakers to use fewer preservatives, particularly those based on organic acids, in intermediate moisture bakery items, early identification of food spoilage is crucial. Large economic losses result from food spoiling because it wastes raw materials or finished goods. The primary issue driving the degeneration of bread goods is microbial spoilage, however physiological spoilage issues including enzymatic spoilage also exist. Most microbes must grow on certain media in order to be detected traditionally, which can take several days from isolation to identification. These techniques provide sensitive, affordable, and qualitative data on the quantity and kind of microorganisms present in a food sample.

Spoilage of Bread

The main product made with flours is bread. The flours used to make the dough go through fermentation, which requires the growth of good microbes. Souring results when this fermentation goes above the necessary thresholds. Proteolytic bacterial growth that is out of control lowers the gas holding capacity needed for dough rising. Moldiness and ropiness are the two main types of bread spoilage. The Bacillus genus, specifically Bacillus subtilis, is the source of the microorganisms responsible for ropiness (formerly referred to as B. mesenteric). However, B. subtilis, B. pumilus, B. cereus, B. licheniformis, B. megaterium (Sorokulova et al., 2003), and B. subtilis can also be the culprits behind ropey bread (Rumeus and Turtoi, 2013). Aerobic, Gram-positive, and able to produce heat-resistant endospores are characteristics of the Bacillus species. These bacteria are typical soil organisms and may contaminate raw ingredients or baking tools. Despite being a food with minimal water activity and generally being regarded as a microbiologically safe product, all types of flour were contaminated with Bacillus species (Yi bar et al., 2012). Because bread is baked at a very high temperature, there are fewer opportunities for microorganisms to survive. Therefore, contamination typically happens during cooling as well as during packing, handling, and environmental factors. These methods are labor-intensive since the common moulds are Rhizopus stolonifera (also known as bread mould), Penicillium expense, Aspergillus Niger, Mucor spp., and Geotrichum.

Types of bread spoilage:

1) Moldiness: The quality of becoming old, stale, or mouldy due to the growth of mould or mustiness. Staleness is the result of losing purity or freshness over time.

2) Ropiness: This bacterial growth-related condition is more common in homemade bread.

3) Chalky bread: Yeast growth is the cause. (2012) Saran raj and Geetha; (2015) Saran raj Penicillium spp. are the most prevalent bread spoiling moulds, while Aspergillus spp. may be more significant in tropical areas (Legan, 1993). Numerous spoilage moulds, such as Penicillium, Aspergillus, Cladosporium, Mucorales, and Rhizopus, have been found in wheat bread. Stolonifera is a prevalent black bread mould among Nigerians. It looks very fluffy and has black sporangia and white cottony mycelium. (2000) Pateras. Table 2 elaborates more on the bread spoilage caused by specific microbes.

Table 2: Microbes responsible for bread spoilage.

Agents	Influencing factors/ species	Properties of The Microbe's Colony	Issues	References
Bacteria	*Bacillus subtilis*	Irregularly shaped.	Rotten odor.	
	Bacillus licheniformis	White or dull in color.	Sticky bread crumps.	
	Bacillus pumilus	Gram positive.	Protein degradation.	
Mold	*Rhizopus nigricans*	Grey in color, fluffy in shape and fast spread.	Mycotoxin.	[1]
	Penicillium expansum	Blue-green in color and slow spread.	Loss of product.	
	Aspergillus niger	Black in color, fluffy in shape and sporehead.	Chalk mold.	

Pathogenicity of *Bacillus sp*.

One of the several groups, Bacillus, has nearly 200 species (Cote et al., 2015). There are numerous recognized species of Bacillus. These strains are rod-shaped, aerobic, gram-positive bacteria that produce endospores. Its whole genome was sequenced in the year 1997. (Du and Web, 2011). High amounts of it are found in the soil, water, and food products, particularly those with plant origins. It is well known that this species is quite active. They are a particularly helpful species because they are known to produce useful enzymes and antibiotics. Additionally, they are probiotics (Schultz et al., 2017) It is generally known that the contamination of bread by Bacillus subtilis results in its ropiness. It is a quick-growing, non-pathogenic bacteria. It is commonly employed as prokaryotic model for investigation of replication, cell wall structure, gene regulation, and metabolism. *B. subtilis* and its many strains have been studied.

Figure 4: Illustration of how ropiness in bread is widely used for fermentative synthesis of industrial antibiotics, enzymes, vitamins, and other value-added compounds (Du and Web, 2011).

Figure 5A: Bacillus subtilis under a microscope.

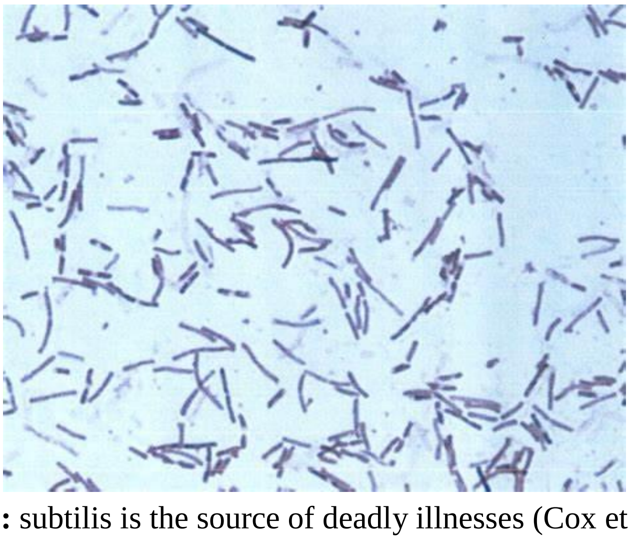

Figure 5B: subtilis is the source of deadly illnesses (Cox et al., 1959).

B. subtilis is everywhere, including soil, water, the air, and even plant remains. The bacteria often only exist as spores and are not physiologically active (Alexander, 1977). It has demonstrated that it can develop. at a range of temperatures, including the body's (Berkeley, 1986). Chills, a harsh cough, and a persistent fever are symptoms of sepsis and pneumonia that are generally caused by B. subtilis. Proteinases hydrolyze the protein in bread flour when it is ropy (gluten). Bacteria that make ropes have the potential to harm humans. Conjunctivitis can be caused by *B. subtilis* (Alloyna, 2011). B. pumilus can result in foodborne disease (Matarante et al., 2004). Bacteremia, peritonitis, gastrointestinal distress, and ocular infection are all brought on by B. licheniformis (Haydushka et al., 2012). An enterotoxin produced by B. megaterium plays a key role in the signs and symptoms of foodborne illness (Lopez and Alippi, 2010). Two classes of toxins produced by B. cereus have been linked to diarrheic (heat-labile) and emetic (heat-stable) toxins (Yi bar et al., 2012). The hydrolysis of starch by amylases promotes ropiness. Ropiness leads to the emergence of a yellow to brown hue, a mushy, gooey surface, and a distinct odor. Bread's softness and stickiness are caused by an extracellular slimy polysaccharide (Pepe et al, 2003). Ropiness is the most noticeable type of bread decomposition after moldiness. It happens in the summertime when Bacillus can flourish because of the favorable climate. The ropiness of bread puts health at risk, and meals containing large amounts of B. subtilis may have negative health effects. Consuming ropy bread results in a significant number of food-borne illnesses in Canada and the UK (Ybar et al, 2012; Rumeus and Turtoi 2013) It is important to avoid eating ropey bread because it might cause symptoms including diarrhea, headaches, nausea, and vomiting (Voysey et al, 1989; Anon 1988). Ropiness typically develops in environments with humid (water activity 0.95) and temperature ranges of 25 to 300 C, which promote spore germination. It should be emphasized that particular storage conditions for temperature, pH, and water activity are necessary as these factors can promote Bacillus spp. spore germination and vegetative cell growth. Rope-causing strains in bread are confirmed by faster amylase and protease growth and production. The traditional process of sourdough fermentation of bread has been chosen to reduce rope deterioration since it causes natural bread's acidification It has been demonstrated that sourdough starter for wheat bread promotes the thermal inactivation of Bacillus subtilis spores (Pepe et al, 2003).

Fig 6: Bacillus growth is depicted in Figure 6 on a strawberry.

The right lung of a Javanese guy who had the aforementioned symptoms and physical evidence of scattered areas of His left lung's infiltration was normal. There was a palpable spleen discovered. It was a case of gangrene of the lungs when B. subtilis was isolated in culture from blood (Bias, 1927). A few factory workers who were used in the production of detergent became the subject of an investigation. Asthmatic symptoms and pulmonary responses were present in some presentations. Additionally, certain allergic reactions with gasping crises that indicated an allergy to the enzyme were obtained. Finally, it was shown that breathing in derivatives of *B. subtilis* containing the proteolytic enzyme can result in permanent lung damage and impairment (Flindt, 1969). Additionally, Bacillus subtilis was identified from animal abortions in bovine and ovine, although it was never determined to be the causative culprit (Logan, 1988). B. subtilis injections were observed to cause soft rot on garlic cloves in the case of plants (Kara rah et al., 1985). Food deterioration is also a result of Bacillus spp (jenson,2014). Sometimes food poisoning with B. cereus and B. licheniformis results in pains in the stomach, diarrhea, nausea, and vomiting. Some Bacillus strains create toxins for insects or cause potato rot. In Bt crops, Bacillus thuringiensus is one example, but *B. anthracis* causes anthrax, which is typically seen in animals and causes high fever, chest pain, and can be lethal if untreated (European Bioinformatics Institute).

The genetic diversity of Bacillus spp. and its capacity to contribute to the ropiness of bread were investigated in an experiment. The bacteria were identified using traditional molecular techniques like 16s DNA sequencing and Random amplified polymorphic DNA (RAPD-PCR). B. licheniformis and B. subtilis were the two organisms most frequently responsible for rope deterioration in bread. Tracking Bacillus strains throughout bread processing and comprehending the importance of several Bacillus spp. in bread spoiling can both benefit from RAPD typing (Sorokulava et al, 2003). Ropey bread can make people sick with a number of conditions, including conjunctivitis, peritonitis, eye infections, and digestive system disorders. To counteract this problem, various preservatives are thus added to bread. It is supplemented with several antimicrobials, including acetic acid and lactic acid (Widyasari et al, 2015).

Control of bacterial spoilage

Ropiness can be avoided via chemical or biological techniques. Since rope-forming bacteria are extremely sensitive to low pH levels, adding chemicals to the dough prevents their growth. Propionic acid, calcium propionate, acetic acid, and calcium hydrogen phosphate are the ingredients that produce bread most effectively. A dose of 1–5 g/kg of flour results in a 3–21-day delay in ropiness. The use of starter cultures of propionic bacteria, such as Propionibacterium shamanic, which exhibit hostile behavior toward Bacillus genus rope-forming bacteria owing to the generation of propionic acid and some compounds with antibiotic properties, is one of the biological techniques. Sourdough, liquid yeast, lactic-acid cultures comprising Lactobacillus plantarum, Lactobacillus brevis, and Lactobacillus ferment are some more biological techniques (Saranraj and Geetha, 2012). Studies on the prevention of ropiness have used antimicrobials such acetic acid, lactic acid, and quaternary ammonium cations (QACs) as well as other methods (Erem et al., 2009). However, acetic acid degrades the baked good's organoleptic properties. As a result, it must be used in conjunction with other antimicrobial agents, such as lactic acid, which could be a different strategy. Although it is frequently used in meat products as humectants and flavour enhancers, lactic acid has not been studied as a bread preservative (Pattison et al., 2004). Low quantities of QACs can stop the growth of bacteria but do not actually kill them. The earlier study (Malek and Malek, 2012) demonstrated the antibacterial activity of QACs against the rope-producing bacterium B. subtilis. Valerio et al. (2012) used molecular techniques together with a quick and cutting-edge technology called FT-NIR (Fourier transform near-infrared) spectroscopy to analyses the variety of spore-forming bacteria isolated from raw materials and bread. They discovered 13 bacterial species, ten of which belonged to the Bacillus and three to the Paenibacillus genus. The most prevalent species, also discovered in ropy bread, was Bacillus amyloliquefaciens. Although B. subtilis and B. pumilus were only represented by a small number of isolates, the screening test for rope production revealed that Bacillus amyloliquefaciens, along with B. subtilis and B. pumilus, were mostly responsible for bread spoiling. A reduced percentage of isolates possibly capable of causing the rope was seen in Bacillus cereus and B. megaterium. But taking into account the large number of B. cereus group isolates found in Valerio et al. (2012)'s study, this bacterial group should also be seen as crucial in rope deterioration (Saranraj, 2015). Chalky bread is another type of bread deterioration brought on by the development of yeast-like fungus Endo mycosis fibuligera and Trichosporon variable. The appearance of white specks that resemble chalk defines this deterioration. Red or Bloody bread is an unusual bread deterioration caused by the bacterial growth Serratia marcescens. This bacterium gives starchy foods a bright crimson color that resembles blood. Additionally, Geotrichum and Neurospora may contribute to the coloring of spoiled bread.

Rhizopus growth on bread

Bread moldiness is seen when it is stored in an environment with high relative humidity and high relative temperature. Depending on the species present and the degree of sporulation variation, different colors of mould growth were seen, including golden yellow, green-grey, and black (Vaghela’s et al., 2011).

Microbial deterioration is mostly caused by mould development. Numerous earlier investigations revealed that different fungi, including Aspergillus, Penicillium, and Fusarium species, develop on bread after bread moulds like Mucor and Rhizopus (Ravi Mannan, 2016). A common flaw in the banking sector is the presence of mould on bread, which reduces productivity by 1-5%.

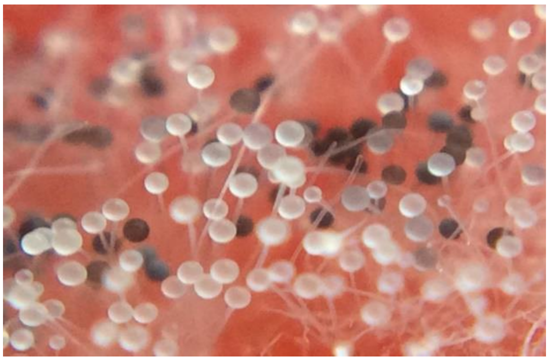

Fig 7: *Rhizopus stolonifera*.

Bread becomes contaminated when mycotoxins form, changing the bread's flavour and color as well as its quality. Because the spores in the flour are killed during baking, mouldy bread is caused by external contamination after baking. Bread contamination can happen throughout the following processes: transporting the bread, cooling it, and storing it, as well as cutting and packaging it. Mold spores may inadvertently reach the bread through the air or through contact with the crates while being stored (Vagelas, 2011).

Spoilage of bread sample with Rhizopus spp.

Based on their physical and cultural traits, the causative fungi were separated and identified. Aspergillus and Penicillium sp. were the next most common causes of bread rotting, followed by Mucor sp. and Rhizopus sp. Therefore, Rhizopus was the widespread fungus discovered on bread (Ravimannan et al., 2016). (Ravimannan et al., 2016). Rhizopus is a member of the fungal family Zygomycetes. It typically flourishes on decomposing and dead materials (Lennartsson et al., 2014). It doesn't have cross walls and makes sporangiospores rather than conidia. The typical bread fungus is Rhizopus Stolonifer. It is one of the significant Rhizopus species (Sylvia et al., 2014). It also goes by the name "black bread"

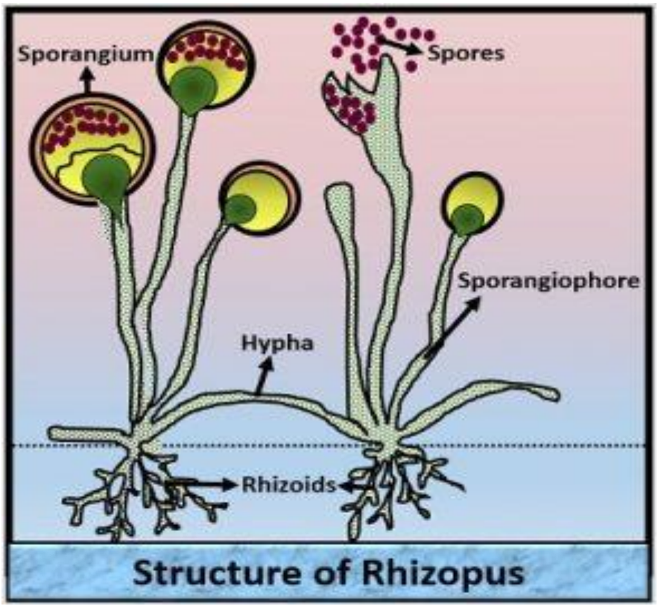

Figure 8: Structure of Rhizopus \s mold.

The moisture-rich area of the (biologyreader.com) plant serves as the catalyst for its first growth. Slice of bread (Muhammad, 2014). With white mycelia and black sporangia, it grows quite quickly. Rhizopus also ruins strawberries, other berries, and vegetables in addition to bread. The most frequent genus that contaminates food is Rhizopus, which is a member of the biological order Mucorales. High moisture levels are necessary for their growth (Bullerman, 2003). Rhizopus spores are water- and airborne-transmissible. It needs a high temperature between 21 and 32 degrees Celsius and a relative humidity of at least 75%. (David, 1992). Lactic acid can be produced by Rhizopus species on synthetic media with inorganic nitrogen sources (Litchfield, 2009). Black bread mould spoils bread by removing nutrients from the bread's surface. It is a kind of heterotrophic mould that has a threadlike appearance. For its growth, it is dependent on carbon sources, particularly sugar and starch. In addition to bread, it is a food supply that is eventually depleted for growth and reproduction and is also present in soft fruits. It is made up of multinucleated, quickly developing hyphae called mycelium. More mycelium is produced during germination when mould spores are dispersed into the environment. Mold that has matured turns black. Within a few days, Rhizopus stolonifera covers the bread's surface and disperses its spores into the atmosphere. The typical range for spore growth is 15–300C.

Fungal contamination of bread

Even before the invention of bread, cereals have long been a significant part of human diets. Sales of bread and other bakery goods have increased significantly during the last few decades. The key components found in bread include proteins, carbs, fats, vitamins, and minerals. Fungus cannot grow as quickly on bread when it is stored in low-humidity environments. Bread contaminated by fungi not only results in financial losses but also the creation of mycotoxin. Along with Rhizopus, Aspergillus, Europium, and Penicillium are also present. Figure 9: Comparison of yeast growth and microbial growth in bread. As a result, filamentous fungal development is more prevalent in bread due to the addition of preservatives to bakery goods and bread (sciencedirect.com) to increase shelf life. Bread deterioration brought on by microbial development ultimately costs both producers and consumers money. On average, 200 million pounds of bakery products are lost each year owing to mould spoilage. Previously regarded as ill man's food, bakery products are now an important component of the human diet (Sheikha and Mahmoud, 2015).

Fig 9: Fungal contamination of bread.

Pathogenicity of Rhizopus

One of the most common postharvest diseases of stone fruits is Rhizopus rot, which is brought on by Rhizopus stolonifer. The illness usually affects ripe fruit, which is more vulnerable to damage and contains more sugar. Fruits become mushy and watery and leak juices with an acidic or fermented odor after 1-2 days of illness. Numerous fruits and vegetables, such as potato, aubergine, watermelon, melon, cucumber, etc., might develop postharvest rots as a result of their presence. Rhizopus is a parasite that only lives in wounds. Rhizopus can enter a plant through fresh wounds, microwounds, and bruising caused by harvesting, handling, and/or insects. There are additional penetration techniques used by Rhizopus stolonifer. R. stolonifer spores need outside food sources to germinate (Baggio, 2015). (Baggio, 2015). Strawberries that have Rhizopus soft rot are characterized by a watery, soft rot that quickly collapses the entire fruit. A thick layer of grey-colored hairy mycelia with black sporangia at their tips covers the diseased fruit, indicating the presence of colonies. Figure 10 on page 13 from the School of Biotechnology and Applied Science depicts soft rot on a strawberry (wikiwand.com) a bacterial and yeast growth on the affected fruit producing a sour odor (Nishijima,1993).

Fig 10: Pathogenicity of Rhizopus in Strawberry

R. arrhizus is the main cause of the uncommon and dangerous disease known as Mucor mycosis or zygomycosis. It mostly affects people with severe malnutrition, diabetic ketoacidosis patients, or patients with weakened immune systems, such as those with HIV/AIDS or certain malignancies. It is possible for the virus to penetrate blood arteries and harm crucial organs including the brain and lungs.

The illness has a 50% fatality rate on average (Petruzello, 2013). There are six different forms of mucormycotic: rare, gastrointestinal, pulmonary, cutaneous, and rhino cerebral (Petrikkos et al., 2012). One-sided facial swelling, headache, nasal or sinus congestion, rapidly worsening black lesions on the upper inside of the mouth, and fever are signs of rhino cerebral mucormycotic. Flu-like symptoms, a coughing fit, chest pain, and shortness of breath are signs of pulmonary mucormycotic. Blisters or ulcers are signs of cutaneous mucormycotic, and the infected area may turn black as well as experience pain, warmth, extreme redness, or swelling close to a wound. Abdominal pain, nausea, vomiting, and gastrointestinal bleeding are signs of gastrointestinal mucormycotic. Disseminated mucormycotic affects severely ill people, and while it is difficult to pinpoint the exact symptoms, it can cause comas if it infects the brain (Spielberg et al., 2005). The vital nutritional value of the chemicals used shouldn't degrade. The application of the following techniques is advised for the efficient decontamination of particular mycotoxins.

Mycotoxin production and their prevention

Due to the involvement of numerous filamentous fungi like Rhizopus, Mucor, and Aspergillus, among others, in bread spoilage brought on by improper handling and sanitation, losses of bakery products attributable to these fungi range from 1.5% to 2.5% depending on the time of year, the type of product, and the processing methods used. Because more bread is being manufactured without preservatives and frequently raw ingredients like bran and seeds are included, it is likely that the incidence of wheat bread spoiling brought on by these fungi has increased over the past several years. These bakery products could be spoiled, which could pose a health risk to consumers and result in a minor food poisoning. It has been proven that eating these goods can cause foodborne illnesses. (Patil, 2019).

Low-molecular-weight natural substances, or tiny compounds, called mycotoxins are created by filamentous fungus as secondary metabolites. These metabolites make up a toxicologically and chemically diverse collection that is only grouped together because their constituents can harm or even kill humans and other vertebrates. Aflatoxin names refer to mycotoxins produced by Aspergillus flavus. Cereals (maize, sorghum, rice, and wheat), oilseeds, spices, and nuts frequently contain aflatoxins. Aflatoxins were categorized by the International Agency for Research on Cancer as a group 1 carcinogen (IARC). Mycotoxins made researchers more aware of the potential for additional mysterious mold. The term "mycotoxin" was soon expanded to cover various previously recognized fungal poisons (such as the ergot alkaloids), albeit some substances in high concentrations are not classified as mycotoxins (Turner et al., 2009; FDA,2013). There are numerous ways to cure mycotoxins, which are dangerous to health. The mycotoxins may transform into less harmful compounds during fermentation (Karlvosky et al., 2016). There are more than 400 known mycotoxins worldwide at the moment. These compounds provide a possible concern to both human and animal health because of their thermal stability. Mycotoxins reduce the marketability of the contaminated products, resulting in significant financial losses (Zinedine et al., 2007). Over 25% of agricultural products worldwide, according to the Food and Agriculture Organization of the United Nations (FAO, 1997), are considerably mycotoxin-contaminated. Monitoring the presence of mycotoxins in food is the best defense against them (Juodeikiene et al., 2012). It is feasible to clean up mycotoxins. Several methods have been developed to remove mycotoxins from foods. Decontamination of mycotoxin-containing food and feed; and prevention or reduction of gastrointestinal absorption of mycotoxin content from eaten food Furthermore, understanding the enzymes involved in the breakdown of mycotoxins brings up some new avenues (Halász et al., 2009). Microorganisms that have been genetically altered are frequently utilized to produce enzymes and in the manufacturing of food. the use of transgenic plants to produce mycotoxin-degrading enzymes by transferring the genes encoding for these enzymes to the plants. Depending on the circumstances, contaminated mycotoxins in meals and feeds should be eliminated, neutralized, or detoxified using physical, chemical, and biological methods.

Control of fungal spoilage

For bakeries, mould spoiling is a significant and expensive problem, making the use of preservatives an appealing way to reduce spoilage and guarantee food safety. However, consumers today do not appreciate additives like preservatives, and the baking business is feeling pressure to use fewer of them. However, it has been demonstrated that reducing preservatives to sub-inhibitory levels might occasionally promote the growth of rotting fungus and increase mycotoxin production (Membre et al. 2001). Mold spores are destroyed during baking, leaving contamination as the cause of spoiling issues. Penicillium sp. makes up 90–100% of the contaminants in wheat bread, but there are also small amounts of Cladosporium and Aspergillus sp., the latter of which is more common in warmer areas.

Molds may develop on a wide range of substrates in the right circumstances Mold growth is the most prevalent type of microbial spoilage in bread processing and, in many circumstances, it is the main determinant of shelf-life. The species of Eurotium, Aspergillus, and Penicillium are the most prevalent and likely most significant moulds when it comes to the biodeterioration of bakery goods. Use of the integrated preservation factors strategy would help prevent fungal deterioration of foods (Sarannraj and Sivaakthivelan, 2016)

Foods with a long shelf life, high quality, and no preservatives are required by consumers. Because additives and contaminants are not perceived as being intrinsic to the food but rather as extras, customers are particularly concerned about them. In a sincere effort to keep bread as natural and as fresh as possible, the bread industry has been working to limit the number of additives and so-called synthetic preservatives since the big Anti-E Number crusade of the mid-1980s. Bread is a perishable good, and its shelf life is typically shortened by a physiochemical deterioration process called staling. This results in a hard, crumbly texture and a loss of the flavour of freshly baked bread. Although the staling phenomena has been thoroughly investigated for decades, it is still unclear from a scientific and technological perspective how staling works. After baking, bread becomes contaminated, and the main factor in bread spoiling is the airborne spread of dust and mould spores. In addition to causing financial losses, bread rotting puts consumers' health at risk, particularly when the bread is infested with mycotoxin-producing moulds. The most widely used methods for preventing bread deterioration include modified environment packaging, irradiation, and the use of preservatives. To meet the rising demand for organic, high-quality products, there is a real need for further safe and effective methods of preventing bread spoiling. The antifungal chemicals produced by LAB and their activity against common bread spoiling organisms have been documented in numerous research over the past ten years (Sarannraj and Sivaakthivelan, 2016).

Bio-preservation to control the spoilage of bread

The most common, useful, and economical method for preventing post-baking contamination is product reformulation, which is used in the bread industry. Lowering the product's aw and pH, which are connected to the microorganisms' shelf life, accomplishes this. To stop bacterial and microbial decomposition, they also utilize chemical preservatives either inside the product or immediately on its surface. Chemical preservatives, according to Sofos J et al. (1981), block microbial metabolism by physically rupturing cell membranes or denaturing cell proteins. The two chemical preservatives that are most frequently employed in bread are propionic acid and its salt. It aids in avoiding bread ropiness and mold degeneration brought on by *B. subtilis* (Rahman et al. 2022).

However, given the potential for developing chronic non-communicable diseases, research into them is ongoing. Bio-preservatives have therefore become a popular remedy for these flaws with the aim of producing "clean label" meals. Bio-preservatives can also be used as organic antifungal agents to stop fungal decay and extend shelf life, lowering threats to the public's health. Due to customer concerns about using chemicals in food, bio-preservatives like lactic acid bacteria, essential oils, or natural nanoparticles are growing in popularity (Rahman et al. 2022).

A good bio-preservative should have the following qualities: a broad antibacterial spectral range, non-toxicity to humans, suitability for lower doses, a slight impact on product pH, no adverse effects on product odor, color, or flavor at the proposed level of use, accessibility in a dry state, higher water solubility, non-corrosiveness, underactivity, and no negative effects on fermentation or bread character (Rahman et al. 2022).

Antimicrobial Activity of Selected Fungal 1solates

Antibacterial activity of different Pan fungal isolates against several known bacterial pathogen. Results show highest inhibition Penicillium extract against Staphylococcus aureus. A zone of inhibition diameter of 16.7 mm was the smallest 10.3mm by Aspergillus oryzae extract. It states that the genus Aspergillus is the most important. Contributions to fungal-derived antibacterial compounds. There is a variation in potential Mushroom extract against bacterial test pathogens. In comparison, Penicillium extract showed greater inhibition Activity against bacteria tested as none were resistant excerpt from him. S. Gram-positive bacteria (*E. coli* and *Pseudomonas*) were more sensitive to mushroom extracts "drugs" over gram-positive ones (*staphylococci* and *staphylococci Bacillus*). Also, excerpts from 3 of the 7 Rhizopus extract was weakly active, thus the fungal isolate showed antibacterial activity against all the bacteria mentioned for *E. coli* only.

Identification strategies for detecting of bacillus Rhizopus species on bread

From several research articles we have come across several studies. Among them the study most common or preferred has been, 16S rRNA sequencing followed by PCR. By 16S rRNA gene sequencing, isolates typical of each rep-PCR profile were initially identified. The 16S rRNA gene was amplified using the universal primers P0 and P16S-1541 because of their positions at the 5′ and 3′ ends of the gene, respectively. Each 50 l reaction mixture included 5 l of the 10 AccuPrime^{TM} Pfx Reaction Mix, 1.25 U of AccuPrime^{TM} Pfx DNA polymerase (Invitrogen, CA, USA), 0.3 M of each primer, and 1 l of genomic DNA. A GeneAmp PCR system 9700 was used to carry out the PCR amplifications. A GeneAmp PCR system 9700 was used to carry out the PCR amplifications. The reaction mixtures were cycled for 30 cycles using the temperature profiles shown below after being preheated to 95 °C for 5 min. 40 seconds at 95 °C, 60 seconds at 53 °C for annealing, 2 minutes at 68 °C, and then a final extension for 10 minutes at 68 °C. Aliquots of the PCR products (2 l) were electrophoresed in 1% agarose gel in TAE buffer and stained with 0.5 g/ml ethidium bromide for analysis. By comparing the size of the amplified DNA fragments to a Gel Pilot 100 bp Plus Ladder (Qiagen GmbH, Hilden, Germany), the size of the fragments was assessed. The PCR products were purified using a Qiagen QIAquick PCR Purification Kit, and their quantities were determined using a NanoDrop Technologies, Inc., Wilmington, USA, ND 1000 spectrophotometer. Using the universal primers P0, P6, P6We16S2R, and P5, the 16S rRNA gene PCR products were sequenced in both forward and reverse orientations.

Bacillus subtilis, Bacillus amyloliquefaciens, and *Bacillus mojavensis* were also identified using partial amplification and sequencing of the gyrA gene using the primer pairs p-gyrA-f/p-gyrA, B amy-gyrA-f/B amy-gyrA, and B moj-gyrA-f/B moj-gyrA, respectively. By employing the primers B pum-gyrB-f and B pum-gyrB-r to amplify and analyse the gyrB gene sequences, B. pumilus and Bacillus safensis were also discovered (Valerio F et al. 2012).

5. Future Directions

From this review it is visualized that 16S rRNA sequencing followed by real time PCR or PCR based methods with a prior enrichment method can be certainly deployed for the successful identification of Rhizopus and Bacillus species involved with bread spoilage.

6. Conclusion

This review elaborately describes the spoilage of bread along with the causative agents responsible for the same. Method for detection of the causative agent for spoilage has also been described elaborately stating the methodology itself in this review. This review is most certainly going to give a future direction to the researchers who are going to work in this field.

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