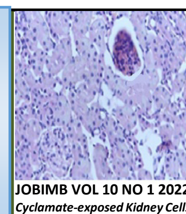
JOBIMB, 2022, Vol 10, No 1, 41-45
https://doi.org/10.54987/jobimb.v10i1.662

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index



Study of Enzymatic Activity of Fungi Isolated from Spoiled Bread Found in Contaminated Soil Around Birnin Kebbi City, Kebbi state Nigeria

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HISTORY

Received: 12th June 2022
Received in revised form: 15th July 2022
Accepted: 25th July 2022

KEYWORDS

Bread
Enzymes
Aspergillus
Penicillium
Rhizopus

ABSTRACT

Enzymes ranging from biochemical substances that are of industrial significance that includes by-products of both primary and secondary metabolites. Ethanol, citric acid, gluconic acid, itaconic acid, amino acid, Vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi. Antibiotics such as penicillin, cephalosporin, fusidic acid and griseofulvin are important secondary metabolites. Fungi are well known as a source of antibiotics but new therapeutic compounds with novel pharmacological activities have been developed in recent years. Loaves of spoilt Bread recruited for this study were collected from different Areas of Birnin Kebbi town and exposed for 7 days in different Areas and are assessed daily. Potato Dextrose Agar (PDA) was used as a culture medium for the isolation. Three fungal species were isolated from Bread samples from contaminated sites using Potato Dextrose Agar. Characterization was based on classical microscopic and macroscopic techniques of color margin and elevation of pure colonies. The consumption of the spores of Fungi that is associated with the spoilage of Bread leads to an increase in Airborne yeasts infections, previous studies had shown that most fungi species that grow on bread stand a major factor of respiratory infection due to their cosmopolitans in the distribution of spores.

INTRODUCTION

Fungi associated with the spoilage of bread lead to an increase in airborne yeast infections. Flour used for the production of bakery products is made from grain that has been grounded to a powdery consistency. Other flour that is most commonly used for breads is wheat grains. The portentous nature of the flour indicates the quality of the bread. An extended time mixing results to the oxidation of the dough, which gives the final product a whiter crumb, instead or cream color preferred by most artisan bakers [1]. Species of *Rhizopus stolonifer* is known as black bread mold [2]. It is a member of *Zygomycota* and is considered the most important species in the genus *Rhizopus*. This fungi specie is one of the most common fungi in the world and has a worldwide distribution of its spores, although it is most commonly found in tropical and subtropical regions [3].

It is a common agent of decomposition of stored foods [4]. Like other members of the genus *Rhizopus, R. stolonifer* grows rapidly, mostly in indoor environments [5]. *Rhizopus stolonifer* is a worldwide distributed species. It is found on all types of mouldy materials. It is often one of the first molds to appear on stale bread [6]. It can exist in the soil as well as in the air. A variety of natural substrata are colonized by this species because

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R. stolonifer can tolerate broad variations in the concentration of essential nutrients and can use carbon and nitrogen combined in diverse forms [6]. In the laboratory, this fungus grows well on different media, including those that contain ammonium salts or amino compounds [6]. However, *R. stolonifer* will not grow on Czapek's agar because it cannot utilize nitrogen in the form of nitrate [6]. Rhizopus lives in hyphae and matured spores.

Fungi associated with the spoilage of bread lead to an increase in airborne yeasts infections. Flour used for the production of bakery products were made from grain that has been transformed into powdery form. Aside the clinical importance, industrial use of fungi is greatly associated with the production of fermented products, including antibiotic enzymes and a range of biochemical. Ethanol, citric acid, gluconic acid, itaconic acid, amino acid, Vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi. Antibiotics such as penicillin, fusidic acid and griseofulvin are important secondary metabolites. Fungi are well known as a source of antibiotics but new therapeutic compounds with novel pharmacological activities have been developed in recent years.

The consumption of the spores of Fungi associated with the spoilage of Bread lead to an increase in airborne yeasts infections, previous studies had shown that most fungi species that grow on Bread stands major factor of respiratory infection due to their cosmopolitans in distribution. An infection caused by this fungus mostly occurs on ripe fruits, such as strawberries, melon and peach, which are more susceptible to wounds and have higher sugar content [7,19]. After a couple of days, the infected fruits become soft and release juices with an acidic odour [7]. When the humidity and temperature are favourable, the mycelial growth occurs rapidly at the surface of the infected fruit and the disease causes the development of long mycelial stolons with black sporangia and spores [7,20]. When the fungus germinates, it produces different kinds of esterases, including cutinase, which help the fungus to penetrate the plant cell wall [7,23]. The disease can also affect other adjacent healthy fruits when distributed by wind or insect activity [8].

The treatment of sweet potatoes with sodium orthophenyl phenol (Stopmold B) and dichloran (Botran W) have effectively reduced storage rot *Rhizopus stolonifer* is an opportunistic agent of disease and hence will only cause infection in people with a weakened immunity [8]. Zygomycosis is the main disease that might be caused by this fungus in humans and while it is not entirely understood yet, this disease is very dangerous and can be fatal [9]. The action of smelling spoiled food may be a source of inhalation exposure to the mold [9,24]. Bakery products, like bread, has become an important staple food in many countries. Cereals and bakery products serve as a valuable source of nutrients in the diet of many people. They provide most of our food calories.

Bakery products provide nutrients such as carbohydrates, proteins, lipids vitamins and minerals. A variety of bakery products are available in the market. Earlier bakery products were considered a sick man's diet or poor man's diet. It has now become the essential food item for a vast majority of the whole population. Bread is made by mixing flour, salt, yeast and other ingredients which are followed by baking. The basic process involves mixing the above ingredients until the flour is made into dough. The dough is baked into a loaf. The dough is made in such a way that will rise easily and be able to give bread of good quality to the consumer.

Yeast is used in the dough which releases CO_2 and the bread becomes spongy. Earlier airborne yeasts were used in making bread. This was done by keeping the dough exposed to air for some time before baking. But the technology has improved bread making to a greater extent in which high energy mixing is involved. Usually, the mold spoilage of bread is due to post-processing contamination. Bread loaves fresh out of the oven are free of molds or mold spores due to their thermal inactivation during the baking process. Bread becomes contaminated after baking, from the mold spores present in the atmosphere surrounding loaves during cooling, slicing, packaging and storage. Most common source of microbial spoilage is due to mold growth. According to the previous studies. Bread molds like *Mucor and Rhizopus* are found to grow first during bread spoilage. This is followed by some other fungi like *Aspergillus, Penicillium and Fusarium* sp. [3].

METHODOLOGY

Study area

This study was carried out around Birnin Kebbi city in Kebbi State, positioned at the north-western part of Nigeria. Predominated by the Gwandu Emirate, With coordinates of 12° 27ˋ13°N4° 12ˋ01°E (TSN,2010).

Study Population

The target population for the study include contaminated sites around Birnin Kebbi city selected bread samples recruited for this study include Bayan kara, Gwandagaji Quarters, Central market, Gesse phase 1, Eastern bypass and Bayan Oando.

Sample collection

Five loaves of bread samples used for this study were collected from different areas including Bayan kara, Gwandagaji Quarters, Central market, Gesse phase 1, Eastern bypass and Bayan Oando were exposed to the laboratory environment for 7 days. The samples collected were brought in sterile polythene bags and transported to the Technology incubation centre quality assurance laboratory in Sokoto state for analysis and were exposed to the laboratory environment for 7 days. All apparatus used in this study were sterilized in a hot air oven at 160 ˚C for 2 hrs. Other equipment was sterilized by autoclaving at 121˚C for 15 mins. Potato Dextrose Agar (PDA) commonly used medium in growing fungi was used in this study. 39g of PDA was dissolved in 100ml distilled water.

Then this medium was sterilized using autoclaved at 121 ˚C for 15 mins. After sterilization, it was allowed to cool down to about 50 ˚C. About 20 ml of the medium was poured into each sterilized petri dish. The PDA medium in the petri dish was allowed to solidify. A gram of bread sample was mixed with distilled water and a homogenate was prepared. The dilution plate method was carried out to enumerate the fungi, The working surface was sterilized using ethanol. 1ml was taken from the above homogenate. Serial dilution was done at the recommended dilution rate i.e 1:10 (1+9). Dilution was done using saline water. Aliquots are drawn for dilution within one min because fungal spores sediment more quickly than bacteria [5]. Dilutions up to 10-4 were carried out. 0.1 ml of the inoculum was added to the surface of the PDA medium and spread evenly over the surface using a sterile spreader (bent glass rod).

The plates were incubated in an upright position at 30 ˚C for 4-5 days. The same procedure was carried out for all the samples. The fungal count was recorded. The different types of colonies were used as inoculates to obtain pure cultures by subculturing in PDA. A small portion of each sub-cultured colony was cut using

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a sterile scalpel. It was placed on a sterile glass slide using sterile forceps. The slide was covered with a cover slip and placed in a Petri dish. A similar procedure was carried out for other fungal colonies as well. These Petri dishes were left at 30 ˚C for 5 days. The coverslips were taken with forceps and placed on slides containing cotton blue. The excess stain was removed and observed under the microscope. The morphology i.e shape, the structure of conidia, conidiophores, pigmentation, the shape of sporangia, and sporangiophores were recorded. The identification was based on the standard keys available.

Statistical Analysis

Different samples from the garages represented the treatments. There were three replications for every treatment. The result was compared using one-way analysis of variance (ANOVA). This was done to establish if differences ($p<0.05$) were significant between individual treatments. The analysis was done using SPSS version 17.

Screening isolate for enzymatic activity

Determination of amylolytic activity

Starch hydrolysis was done using the method of [7]. Starch is a high molecular weight polymer and is hydrolysed by the enzyme, amylase starch detection of the hydrolytic activity following growth period was made by the medium. Starch in the presence of iodine impacts a blue-black colour to the medium, indicating the absence of the enzyme. When starch has been hydrolyzed, a clear zone of hydrolysis surrounds the colonies hence a positive test.

Determination of proteolytic activity.

To detect protease activity, isolates were cultured on PDA media containing the following in grams per litre nutrient broth 8.0 glucose and agar 18.0 and then pH adjusted to 8.0 PDA media was used for the protease activity. The media was supplemented with 1% skimmed milk as sole carbon source. The isolates were inoculated and incubated at 30^0c for 7 days and protease production by isolates was noted by the presence of a clear halo around colonies [10].

Determination of lipolytic/esterase production

For determining lipase/ esterases production, the isolates were cultured on basal media containing 1% KH_2PO_4, O.O1% $MGSO_4.7H_2O$. 0.005%, $CaCl_2..2H_2O$ 1% NaCl and 1% Na_2CO_2. Supplemented with 1% olive oil (domestic grade) as the sole carbon source. The medium was then inoculated by spotting three isolates per plate and then incubated for 7 days at 30 ºC. The media was observed for zones of precipitation of calcium crystals around each isolate. Lipase /esterase production was indicated by the precipitation of calcium Crystals around the colonies [10].

Determination of cellulolytic activity

Cellulose production was determined by using the media containing 7.0 g KH_2PO_4, O.O1% $MGSO_4$. $7H_2O$, 1.0 g $(NH_4)_2SO_4$, 0.6 g. yeast extract, 10 g microcrystalline cellulose and 15 g agar per litre. The plates were inoculated and incubated at 30 ºC for 7 days. for best viewing of the clear halo, the plates were stored at 50 ºC for one night after 7 days of incubated [10, 19].

RESULTS AND DISCUSSION

Out of 6 bread samples that were used for this study, three fungal species were isolated from bread samples gotten from contaminated sites using Potato Dextrose Agar (**Table 1**). Characterization was based on classical microscopic and macroscopic techniques of color margin and elevation of pure colonies. These isolates were able to grow between 3-7 days of incubation. The isolates exhibited different colony characteristics. Three isolates had curled margins, two had entire margins while the rest were undulate and filamentous. The isolates had raised fluffy elevation. The reverse colour was mostly creamish while the observation varied for each isolate (**Table 2**). The consumption of the spores of Fungi that is associated with the spoilage of bread leads to an increase in airborne yeasts infections, previous studies had shown that most fungi species that grow on bread stand a major factor of respiratory infection due to their cosmopolitans in the distribution of spores.

Table 1. Cultural characteristics of fungi isolated from bread in contaminated sites (area).

Dilution	Isolated identity	Colour (TOP)	Colour (Bottom)	Margin	Elevation
4/10	*Penicillum* spp.	Dark green	Cream green	Entire	Raised Fluffy
5/10	*Asperigillus* spp.	Pinkish brown	Cream green	Curled	Raised
4/10	*Rhizopusw* spp.	Cream white	Cream white	Curled	Raised
5/10	*Asperigillus* spp.	Green	Cream	Curled	Raised
4/10	*Rhizopus* spp.	Cream white	Cream white	Curled	Raised fluffy
5/10	*Asperigillus* spp.	Cream white	Cream	Curled	Raised

Table 2. Fungi isolated from different area with cultural characteristics.

Sites (area)	Dilution factor	Cultural characteristics	Fungi isolates
Bayan kara	4/10	Green colored colonies brush like appearance formed of chains spores, non septate branch hyphal enlarged at the apex to form conidiosphores	*Penicillum* spp.
	5/10	Black powdery colonies , conidiosphores are smooth and colourless and turned dark toward vesicles	*Asperigillus* spp.
Central market	4/10	Uptight Sporangiophores with colonies columnar , nonseptate hyphal connected by stolon and Rhizopus, dark pear shape sporangium hemispherical columella	*Rhizopus* spp.
	5/10	Black powdery colonies and Conidiosphores are smooth	*Asperigillus* spp.
Gesse phase 1	4/10	Dense cottony growth with sporangiophores	*Rhizopus* spp.
	5/10	Cottony growth with sporangiosphores	*Rhizopus* spp.

Table 3. Production of extracellular enzymes by fungal isolates.

dilution code	isolate identity	amylase	protease	lipase esterase	cellulase
4/10	*Penicillum* spp.	+	+	+	+
5/10	*Aspergillus* spp.	+	+	+	+
4/10	*Rhizopus* spp.	+	+	-	+

Key: (+) Denotes hydrolysis and (-) Denotes no hydrolysis

The outcome of this study shows consistency with similarity from those obtained by [2,21]. And only test tubes having higher microbial growth were for the analysis and the ability of the fungal isolates to produce amylase, cellulose, protease and lipase enzymes, indicates their ability to biodegrade enzymes containing substrate were assessed. Industrial use of fungi is

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greatly associated with the production of fermented products after microbial metabolisms, including antibiotic enzymes and a range of biochemical substances that includes Ethanol, citric acid, gluconic acid, itaconic acid, amino acid, Vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi. Antibiotics such as penicillin, the cephalosprins, fusidic acid and *griseofulvin* are important secondary metabolites [11,22].

Three isolates namely *Aspergillus* spp*.,* *Penicillum* spp*.,* and *Rhizopus* spp*.,* were recovered from bread isolated from contaminated sites. The presence of these fungi in bread samples indicated that, the isolates were able to exist in the bread while those that could not survive being eliminated by the unfavorable conditions. The fungi load (count) increases progressively as the period of storage increased. After seven days of incubation, the highest fungal count for all examined samples. The isolated organisms are *Aspergillus* spp*.,* *Penicillum* spp*.,* and *Rhizopus* spp. These findings are similar to that of [1]. But isolates such as Mucor spp., and *Fusarium* spp. that were among the isolates in [4,20]. Were absent in this study. All isolates were positive for amylase hence was hydrolyzed. protease production for all isolates was observed and so skim milk was hydrolyzed. One was negative for lipase/esterase production while the rest two were positive.

This shows that the six positive isolates hydrolyzed the olive oil while the rest two could not. All isolates tested positive for cellulose and so cellulose was well hydrolyzed (**Table 3**) shows hydrolysis of skim by *Aspergillus flavus* and hydrolysis of starch by *Penicillum* spp. The radial growth for the fungal isolates under different pH. The results of the present study are partly consistent with those obtained by [13].

The ability of the fungal isolates to produce amylase, cellulose, protease and lipase enzymes, demonstrate their ability to biodegrade enzymes containing substrate. The main industrial use of fungi has been associated with the production of fermentation products, including antibiotic enzymes and a range of biochemical. Ethanol, citric acid, gluconic acid, itaconic acid, amino acid, Vitamins, nucleotides and polysaccharides provide examples of primary metabolites produced by fungi. Antibiotics such as penicillin, the cephalosprins, fusidic acid and griseofulvin are important secondary metabolites [11,24].

Fungi are well known as a source of antibiotics but new therapeutic compounds with novel pharmacological activities have been developed in recent years [12]. One such example is the cyclosporin first isolated from *Tolypocladium inflatum* in 1979 an antifungal. Cyclosporine is currently the most widely used drug for preventing rejection of human organ transports [13]. After mass production of the fungal isolate recovered, the fungus can be grounded and formulated with adequate carriers or extenders either in the powdered or liquid form using tween 80 which enhance the degradation process. Fungi are acidic friendly; they colonise an environment that is reach in acidity. Raw materials used for the Bread making are mostly acidic in nature as one the intrinsic constituents that enable the growth of black mold [14,22]. The effect of moisture and temperature on growth and spore germination in some fungi, they form mycota as they secrete mycotoxcins as organisms that form mycota, they are cosmopolitan in distribution, as they colonised environment that is inhabitable by other organisms hence, they possess the ability of secreting different types of enzymes to permit their survival most especially in acidic environment with moisture [15,16,20]. The preservative agents use for conservation of bread for long time usage is added the raw materials [14,17,18].

CONCLUSION

The 6 bread samples from contaminated sites which were used for this study, three fungal species were isolated from Bread samples from contaminated sites using Potato Dextrose Agar. Characterization was based on classical microscopic and macroscopic techniques of color margin and elevation of pure colonies. This isolates where able to grow between 3-7 days of incubation. The isolates exhibited different colony characteristics. Three isolates had curled margin, two had entire margin while the rest were undulate and filamentous. The isolates had raised fluffy elevation. The reverse colour was mostly creamish while the observe varied for each isolate. The optimum growth temperature range for the three fungi was 40c. The optimum growth for the three isolates was observed. The three fungi were also screened for extracellular enzymes such as amylase, protease, lipase/ esterase and cellulose enzymes, demonstrating their ability to biodegrade oil containing substrate. This study will contribute to the database on locally available fungal diversity and their ecology and also increases knowledge of the fungi isolated from Bread and their enzymatic process in Birnin kebbi town.

CONCEPTUALIZATION

J.G., A.O; and D.N.B; methodology, J.G., A.O; and H.I; software, J.G. and D.N.B; validation, J.G., A.O, and H.I; writing—original draft preparation J.G; writing—review and editing, J.G and A.O. Supervision, J.A.M; project administration, J.G; funding acquisition, J.G

ACKNOWLEDGMENTS

The first author wishes to acknowledge the support of A.O and B.J by offering an insightful idea to conduct this research work.

CONFLICT INTEREST

Authors have declared no conflict of interest.

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