Inhibitory Effect of Garlic (*Allium sativum* L.) Against Bread Mold and Its Influence on the Quality of Yeast-Leavened Bread

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***Abstract*—Bread spoilage contributes to the food waste problem in the Philippines. This study was conducted to optimize the levels of garlic, sugar and fermentation time of yeast-leavened bread; determine its sensory quality, physico-chemical properties and microbial quality; isolate mold responsible for the bread spoilage; characterize the isolate and assess the inhibitory potential of garlic against the pure culture. Three levels of garlic (1.5%, 2.5% & 3.5% (w/w), sugar (15%, 20% & 25%) and fermentation time (120, 180 & 240 minutes) were the variables considered in the optimization. The optimum combination of the bread is at 3.2% w/w garlic, 19.0% sugar and 125 minutes fermentation time. Significant changes (p≥0.05) on the pH from 6.17 to 5.73 and moisture content from 41.24% to 40.33% were observed on the optimum treatment during storage. Higher microbial load was noted in the control sample (5.7x105CFU/g & 4.5x105 CFU/g for Total Plate Count, & Yeast and Mold Count, respectively), in the 8th day as compared to the optimum with 6.0x102CFU/g and 2.5x103CFU/g. The mold responsible for the spoilage of the bread was identified to be Penicillium sp. The widest zone of inhibition (7mm) was observed at 3.5% garlic level.**

***Index Terms*—bread, garlic, inhibition, fermentation, sugar**

I. Introduction

Food waste is a serious problem not just in the Philippines but in other countries as well. Bread spoilage contributes to the existing problem. Mold spoilage is one of the main causes of bread waste. In addition to food waste and economic losses, it can cause food safety problems due to aflatoxin production.

Bakery products are important staple foods in most of the countries. Bread is one of the most common bakery products that have been part of one's regular food intake. Packed bread products are now on trend which is of great convenience for household members. However, extending the shelf life of these packed bread products is the common problem a bakery industry is encountering. Because of its ingredients, bread is so favorable for the growth and proliferation of microorganisms.

Various microorganisms are found to cause the spoilage of bread, especially the molds. Mold spoilage of bakery products is a serious and costly problem for bakeries and use of preservatives is therefore an attractive means to diminish spoilage and ensure safety [1].

The common microorganisms responsible for the spoilage of bread are *Aspergillus niger*, *Aspergillus flavus* and *Rhizopus stolonifer*. Among these three, the most destructive and common species in bread is Rhizopus stolonifer, generally known as "black bread mold". Most often, the growth of mold initiates with the moisture abundant part of a bread slice, particularly the crease [2].

Preservatives help the reduction of food waste through inhibition of molds in bread. Among these preservatives are propionic and sorbic acid or their salts which have been shown to increase the shelf life of bakery products. Propionic acid and calcium propionate are usually employed at concentrations of 0.1 and 0.2 percent respectively. At these levels, molds can be inhibited for 2 days or more [3]. Propionates are effective against a broad spectrum of molds.Theyhave limited effectiveness against bacteria but one significant exception; they do retard the growth of Bacillus mesentericus which is the organism responsible for "rope" in bread and other yeast leavened products [4]. Today, most consumers are health conscious that they would comprehend the ingredients on the product they are eating. The need for application of natural ingredients on food product has increased.

*Allium sativum L.*or commonly known as garlic, is a vegetable species that can be classified as either a food or a medicinal herb. When crushed, A. sativum yields allicin, an antibiotic and antifungal compound (phytoncide). Fresh or crushed garlic also has enzymes, B vitamins, proteins, minerals, saponins, flavonoids, and Maillard reaction products [5].

Dimić *et al.*, 2009 reported that garlic extract at different concentrations inhibited *A. tamari, P. commune, P. implicatum* and *E. nidulans*. Garlic was most effective

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against E. nidulans and P.implicatum with a hundred percent inhibition at 1% concentration [6]. The effectiveness of garlic extract was also demonstrated by Capangpangan (2002) in preventing growth of aflatoxin-producing *A.flavus*.

With the favorable results on the inhibitory potential of garlic, the application of this commodity on food products such as bread is necessary. The anti-fungal activity of garlic against bread mold in yeast-leavened bread at different concentrations and at different bread processing conditions was assessed.

Generally, this study determined the inhibitory effect of garlic against bread mold in yeast-leavened bread. Specifically, the study aimed to screen the different variables that affects the processing conditions of yeast-leavened bread employing Placket-Burmann (PB); optimize the garlic level, fermentation time and sugar levels for yeast-leavened bread production; determine the sensory quality of the yeast-leavened bread; determine the physico-chemical property (pHand moisture content) and microbial quality of the yeast-leavened bread; isolate and purify the mold responsible for the yeast-leavened bread spoilage; characterize the mold isolate responsible for the yeast-leavened bread spoilage; and assess the inhibitory potential of garlic against the mold isolate pure culture.

II. Materials and Methods

A. Garlic Preparation

The garlic was washed, sanitized (20 ppm) and peeled. It was placed on a garlic crusher for few minutes or until cut into small pieces.

B. Processing of Bread

The bread was prepared from hard wheat flour following the method of Muhammad et al.,(2014) but with some modifications. Wheat flour, sugar, salt, yeast, shortening, skimmed milk and water were mixed together. Instead of a mechanical mixer, manual mixing was done. The mixed dough was placed on a stainless basin subjecting to fermentation process at room temperature. After the required fermentation time, garlic was added into the fermented dough and further manual mixing was employed in order to develop the gluten. The developed dough was cut into portions each having the same mass, molded by hands and kept in baking pans that has been greased with oil. These dough portions were allowed to proof. The molded loaves were baked in an oven at 200 ℃, depan, cooled, sliced and packed in polyethylene bags.

C. Sensory Evaluation

Sensory evaluation using quality scoring in combination with 9-point Hedonic scale was carried out to evaluate the sensory attributes of the different treatments. An Incomplete Block Design (IBD) as laid out by Cochran and Cox (1957) was used during the presentation of the different treatments since fifteen treatments are too many for each panelist to evaluate. The set plan (Appendix B) of t=15, k=7, b=15, r=4, λ=3, E=0.92, Type II was followed where t refers to the number of treatments, r the number of replications based on the IBD, b the number of blocks and E the efficiency factor. The set plan was replicated four times to get 60 panelists with 28 panelists evaluating per treatment.

D. Experimental Treatments

TABLE I. Experimental Combinations of Garlic Levels, Fermentation Time and Sugar Levels in Yeast-Leavened Bread

TREATMENT	GARLIC LEVELS (%)	FERMENTATION TIME (MINS)	SUGAR LEVELS (%)
1	3.5	240	25
2	3.5	240	15
3	3.5	120	15
4	3.5	120	25
5	1.5	240	25
6	1.5	240	15
7	1.5	120	15
8	1.5	120	25
9	2.5	240	20
10	2.5	180	15
11	2.5	120	20
12	2.5	180	25
13	3.5	180	20
14	1.5	180	20
15	2.5	180	20

E. Optimization Experiments

Results in the sensory evaluation was subjected to Response Surface Regression (RSREG) analysis using Statistical Analytical Software version 9 (SAS 2008) in the analysis of the sensory quality and acceptability for all formulations of the product. The Statistica 8.0 software was used for the graphical presentation of the response surface plots.

F. Moisture Content

Determination of moisture content by oven drying was done following the standard protocol set by AOAC, (2012).

G. pH

Five grams of the sample was homogenized in 25ml distilled water. It was then analyzed by a handheld pH meter (HM Digital,China) that has been calibrated using pH buffers of 4.0 and 7.0 (BOECO, Germany).

H. Microbial Analyses

1) Isolation of molds

The yeast-leavened bread with garlic was allowed to stand for few days until visible mold growth were noted. One gram of the moldy bread sample was collected and the predominant mold colony was isolated for further test. Serial dilution and pour plating were done in order to isolate the mold colonies. Plates were incubated for 3-5 days at 30-35 ℃ until visible mold colonies were observed.

2) Purification of molds

Mold growth on solid media were examined after incubation. The predominant mold colonies were transferred into the acidified and solidified PDA on plates by streak plating. The plates were incubated for 3-5 days at 30-35 ℃. Streak plating was repeated until the mold isolate was purified and transferred to an agar slant.

3) Identification of Molds

Cultural Characteristics.A series of dilution from pure culture of mold isolate was prepared. These were aseptically plated in an acidified Potato Dextrose Agar (PDA) and plates were incubated for 3-5 days at 28-32 ℃. Visual observation was done after the incubation period to determine the cultural characteristics of the organism.

4) H. D. Morphological Characteristics.

Agar block technique was done to determine the morphological characteristics of the pure culture. Melted acidified Potato Dextrose Agar (PDA) was aseptically poured into sterile petri plates and was allowed to solidify. With the use of a sterile scalpel, the solidified PDA was cut into 1cm2 .The cut up agar blocks were transferred to sterile glass slides. A sterile needle was dipped into the dilution and was carefully touched at the four sides of the agar block. A sterile cover slip was placed on top of the agar block and the slide was kept inside a sterile plate suspended by a bent stirring rod. The plates were then be incubated at 30-35 ℃ for 3-5 days. The cover slips were removed and transferred to sterile glass slides with a drop of lactophenol and covered with a sterile cover slip. The slides were viewed under a microscope in Oil Immersion Objective (OIO). Morphology of the isolate were evaluated and properly documented. To confirm the identification of the isolate, Prescott and Duns (1982) and Gilman (1957) was used as cited by Capangpangan (2002).

5) H. E. Inhibitory Potential of Garlic Against Bread Mold

A loopful of mold pure culture was transferred to a buffer solution and was mixed. One mL of the dilution was pipetted into the sterile liquefied acidified PDA on a petri plate, swirled and was allowed to solidify. A sterile filter paper disc (30 mm diameter) previously soaked in garlic concentration (1.5%, 2.5% &3.5% w/v) was placed at the center of the plate. Plates were incubated at 30-35 ℃ for 3-5 days. Inhibitory potential of garlic was be evaluated by measuring the zone of inhibition.

6) H. F. Colony Counting

Serial dilution and pour plating method was employed to determine the microbial count of the sample using the Plate Count Agar (PCA). A milliter of the sample was pipetted out into a 9 ml buffered dilution blank. This provides a dilution of 10-1, with another sterile pipette, one ml of the 10-1 was pipetted out to get a 10-3 dilution. The process was repeated until 10-7. One ml of each of the dilution were pipetted out into each petri dishes containing fifteen ml of culture media cooled to 45 ℃. It was then mixed and allowed to solidify. The petri dishes were incubated in an inverted position at room temperature. Colony count ofthe sample was calculated after48 hours of incubation using the following formula.

$$\text{CFU/g} = \frac{\text{Average no. of colonies x dilution factor}}{\text{Volume plated in ml}}$$

III. Results and Discussions

A. Sensory Evaluation

Table II presents the summary of parameter estimates of sensory acceptability of all sensory attributes of the bread. The crust color, flavor, and texture of the yeast-leavened bread were not significantly affected by the garlic levels, sugar levels and fermentation time. However, the interaction of fermentation time and sugar level significantly affected the crumb color acceptability. On the other hand, the aroma acceptability was significantly affected by the fermentation time and with its interaction with the garlic level. Moreover, the taste, aftertaste and general acceptability were found to be affected by the quadratic effect of fermentation time.

B. Optimization

At constant 20% sugar level, the optimum formulation was found to be at 120-136 fermentation time and 3.0% to 3.4% garlic level as illustrated in Fig. 1. Considering this formulation, only treatment 3 was found to be within the shaded region among the 15 treatments used in the experiment.

Fig. 1b presents the graphical representation of superimposed contour plot on sensory attributes at constant 180 minutes fermentation time. From this figure it can be pointed out that the potential optimum formulation for the production of garlic loaf bread was 18% sugar level and 3.0 to 3.5 % garlic levels. The region was limited by the taste and general acceptability.

Shaded region in Fig. 1c demonstrates the possible optimum formulation for the product at constant 2.5% garlic level. The combination of 20.5% sugar and 122 minutes fermentation time was found inside the shaded area. This is nearly the same as to the formulation of treatment 11. Any combination found inside the shaded optimum region can be used in producing the yeast-leavened bread To control the consistency of the quality of the product one (1) point should be used for processing purposes and the combination points found inside the shaded region could be used as reference or critical limits should problems occur during production like deviations from the quality standard. For processing and quality control purposes, the combination of garlic, sugar and fermentation time was set at 3.2%, 19% and 125 minutes, respectively.

TABLE II. Summary of Parameter Estimates for the Response of Sensory Acceptability of all The Sensory Attributes of Yeast-Leavened Bread

PARAMETER	CRUMB COLOR	CRUST COLOR	AROMA	FLAVOR	TEXTURE	TASTE	AFTERTASTE	GENERAL ACCEPTABILITY
Intercept	10.47*	9.08*	14.28*	9.20*	11.66*	2.70*	9.70 *	7.84*

GL	-0.02^{ns}	0.42^{ns}	0.46^{ns}	0.42^{ns}	0.72^{ns}	0.74^{ns}	0.65^{ns}	0.89^{ns}
$(GL)^2$	-0.04^{ns}	0.25^{ns}	0.67^{ns}	0.66^{ns}	0.84^{ns}	0.38^{ns}	0.85^{ns}	-0.12^{ns}
FT	-0.01^{ns}	0.72^{ns}	0.01*	0.19^{ns}	0.10^{ns}	0.19^{ns}	0.09^{ns}	-0.02^{ns}
$(FT)^2$	0.00^{ns}	0.25^{ns}	0.06^{ns}	0.08^{ns}	0.05^{ns}	0.27*	0.04*	0.00*
SL	-0.24^{ns}	0.95^{ns}	0.35^{ns}	0.60^{ns}	0.29^{ns}	0.80^{ns}	0.97^{ns}	-0.03^{ns}
(SL)2	0.01^{ns}	0.92^{ns}	0.50^{ns}	0.43^{ns}	0.24^{ns}	0.97^{ns}	0.77^{ns}	0.00^{ns}
(GL)(FT)	0.00^{ns}	0.48^{ns}	0.01*	0.75^{ns}	1.00^{ns}	0.62^{ns}	0.42^{ns}	0.00^{ns}
(GL)(SL)	0.00^{ns}	0.96^{ns}	0.22^{ns}	0.75^{ns}	0.92^{ns}	0.32^{ns}	0.87^{ns}	-0.01^{ns}
(FT)(SL)	0.00*	0.21^{ns}	0.36^{ns}	0.39^{ns}	0.84^{ns}	0.62^{ns}	0.36^{ns}	0.00^{ns}

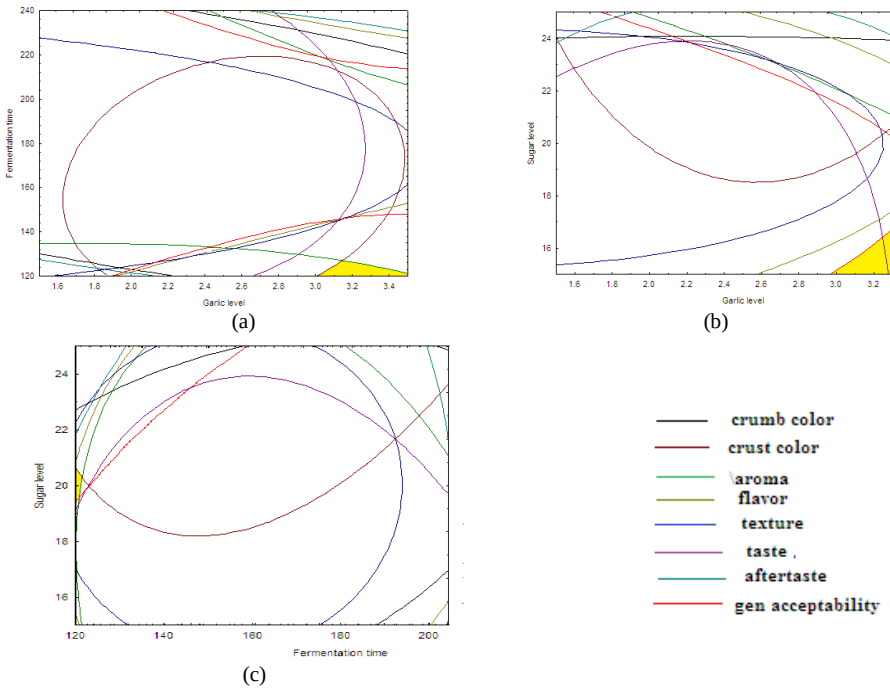


Figure 1. Optimum region (shaded) for Yeast-Leavened Bread obtained by superimposing contour plots of sensory acceptability ≥7.25 with constant (a) sugar level, (b) fermentation time and (c) garlic level

C. pH and Moisture Content

Table III summarized the mean values for the Analysis of Variance (One-Way ANOVA) of the pH and moisture content of the product during storage and a post-hoc analysis using Least Significant Difference (LSD) test was done in order to compare the significant means. The result shows significant changes on the pH from 6.17 to 5.73 and moisture content values from 41.24% to 40.33% were observed during storage. Although there was a slight increase in the moisture during the 1st, 5th and 6th day, it can still be noted that there was a decrease in moisture content from the time after baking until on its 8thday of storage. The moisture content results were higher as compared to that reported by Latif *et al*., (2005) in which moisture of bread from wheat flour with different additives ranged from 16.75 to 37.13% evaluated for 10 days of storage.

In a similar study, decrease in the moisture of freshly baked bread was observed by Muhammad *et al*., (2014). He reported that bread crumb moisture decreased sharply with time. The crumb of freshly baked bread contained about 47% moisture. However, during 2 hours of cooling the moisture dropped to 41%. Fik *et al*. as cited by Muhammad *et al*. (2014) also reported that the moisture content of calcium enriched bread decreased by 9% in 3 days of initial storage.

TABLE III. ANALYSIS OF VARIANCE (ONE-WAY) AND LSD TEST FOR THE MEAN PH AND MOISTURE OF THE PRODUCT DURING STORAGE

DAY	pH	MOISTURE
0	6.17^{A}	41.24^{DE}
1	5.97^{B}	43.83^{A}
2	5.88^{BC}	42.02^{CD}
3	5.85^{C}	41.25^{DE}
4	6.10 ^{A}	41.71^{CD}
5	5.62^{D}	43.70 ^{A}
6	5.88^{BC}	43.01^{AB}
7	5.35^{E}	42.42^{BC}
8	5.73^{D}	40.33^{E}

The initial pH value of the bread was 6.17 which gradually decreased to 5.73 during 8 days of storage. The statistical analysis (Table I) showed that the treatments and storage intervals had a considerable ($p< 0.05$) effect on pH of the bread samples through storage at ambient temperature. In a similar study, Shin et al. as cited by Muhammad *et al*. (2014) observed pH values of breads from6.28 to 6.08. It was further documented that change in pH of bread was due to the fermentation process. Molds show the high amylase activity (3.9mg/l) at pH 6.

D. Microbial Analyses

Microbial analysis was carried out to the optimum formulation and the control (no garlic content) in order to monitor the potential of garlic as a natural mold inhibitor in bread. Table IV and Table V show the total plate count and yeast and molds results, respectively. It can be clearly observed that higher microbial load can be noted in the control sample with 5.7x105 Colony Forming Unit per gram (CFU/g) TPC, and 4.5x105 CFU/g Yeast and Mold Count in the 8th day as compared to the optimum with 6.0x102CFU/g and 2.5x103CFU/g Total Plate Count and Yeast and Mold Count, respectively. Considering the microbial analysis of the optimized bread formulation as well as its physico-chemical properties, the addition of garlic has extended the shelf life of the bread for four days as compared to the control sample.

Muhammad (2014) reported similar results, with an initial total plate count of $7x10^2$ at 0 day on the control sample (no turmeric and ginger) and $5x10^1$ on the sample with 1.5% ginger + 1.5% turmeric the microbial count of bread as affected by the combination of ginger and turmeric against *Rhizopus stolonifer* as natural mold inhibitors.

The microbial count of the optimum corresponds to the standard plate count of <200,00 CFU/g and <1000 CFU/g yeast and mold count (WQA Manufactured Food Standard, 2012). This means that the optimum product was still within the standard count until 8th day while the control sample was only at 4th day.

TABLE IV. TOTAL PLATE COUNT OF THE OPTIMUM FORMULATION AND CONTROL

DAY	OPTIMUM(CFU/g)	CONTROL(CFU/g)
0	$4.5x10^1$	$1.8x10^1$
1	$11x10^1$	$1.5x10^1$
2	<1	$1.5x10^3$
3	$5.0x10^2$	$9x10^3$
4	$7x10^1$	$15x10^4$
5	$2.9x10^2$	$2.5x10^5$
6	$1.0x10^1$	$4.5x10^{\backslash 5}$
7	$3.4x10^2$	$3.7x10^{\backslash 5}$
8	$6.0x10^2$	$5.7x10^5$

E. Cultural Characteristics

In order to identify the genus of the unknown molds isolated from the yeast-leavened bread containing garlic, its features and characteristics were examined. Table V and Table VI summarized the cultural and morphological characteristics of the mold isolate. The color of young colonies is white and turned into grayish green when mature. Its shape is round and its growth is rapid. Young colonies were visible after 2 days of incubation and become grayish green on its 3rd or 4th day of incubation.

F. Morphological Characteristics

The morphology of the unknown pure culture of molds exhibited a septated hypha and a clear and well developed mycelium. The type of spore is conidia having a round to ovoid shape and a brush-like structure can be distinguished. Its conidiophores are branched. Fig. 1 shows the photograph of the molds under the microscope under Oil Immersion Objective (OIO).

Based on the characteristics and features of the mold isolate, the microorganism was identified to be of the genus *Penicillium*. This culture was used as test organism in determining the inhibitory potential of garlic with the same concentration of that when applied in bread production. According to Visagie *et al*., (2014) conidiophores character is of great taxonomic importance of *Penicillium sp*. The conidiophores branching patterns were traditionally used in the classification of *Penicillium sp.* The conidiophores range from being simple to very complex with multiple levels of branching resulting in overall symmetrical or asymmetrical patterns. Navi *et al*. (1999) isolated several fungi on sorghum grain. The fungi were identified through photomicrography. *Penicillium sp* is readily recognized by its brush-like structure bearing long, well-defined columns of conidia.

TABLE V. YEAST AND MOLDS COUNT OF THE OPTIMUM FORMULATION AND CONTROL

DAY	OPTIMUM(CFU/g)	CONTROL(CFU/g)
0	<1	$15x10^1$
1	<1	$2x10^1$
2	$3.5x10^1$	$5x10^1$
3	$2.0\ x10^1$	$1.3x10^1$
4	$1.9x10^1$	$2.3x10^2$
5	$2.8x10^1$	$1.8.0x10^3$
6	$4.5x10^1$	$2.1x10^5$
7	$1.0x10^3$	2.0^5
8	$2.5x10^3$	$4.5x10^5$

TABLE VI. CULTURAL CHARACTERISTICS OF THE UNKNOWN MOLDS

FEATURES	CHARACTERISTICS
Color	White when young and becomes grayish green when matured
Shape of Colony	Round
Appearance of colony	Velvety; colonies are grayish green at the center and whitish at the periphery
Color produced on culture media	Yellowish

TABLE VII. MORPHOLOGICAL CHARACTERISTICS OF THE UNKNOWN MOLDS VIEWED UNDER THE MICROSCOPE

MORPHOLOGY	CHARACTERISTICS
Hypha	Septated
Mycelium	Clear; developed
Type of Spores	Conidia and with brush-like spore-bearing structure
Conidiophores	Branched
Shape of Conidia	Round to Ovoid

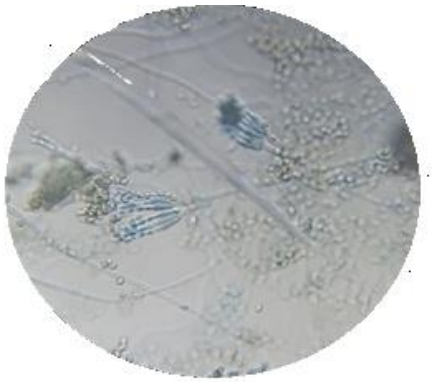


Figure 2. Microscopic examination of isolated mold from yeast leavened bread under 1000X magnification

G. Inhibitory Potential of Garlic Against the Test Organism (Penicillium sp)

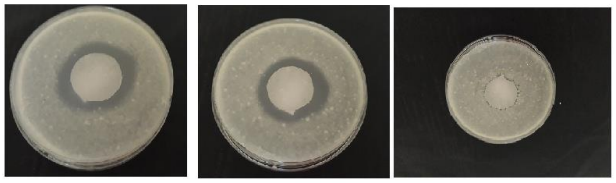
The garlic possesses an inhibitory potential against the test organism (*Penicillium sp*) as shown in the results of its zone of inhibition (Table VIII & Fig. 3). It can be clearly observed that wider zone of inhibition after 3 days of incubation was noted with the increasing level of garlic. No inhibition was noted at 1.5% garlic level. Similar results were reported by Capangpangan (2002) in which the zone of inhibition of *Aspergills flavus* was wider as the garlic extract concentration increases. In the work of Dimic et al. (2009), the extract of garlic only partially (25.4% and 26%) inhibited the growth of *A. tamarii and P. commune*, respectively. However, it inhibited completely (100%) the growth of *P. implicatum* and *E. nidulans* at the doses of 0.5 and 1% concentration.

Allicin is the component of garlic released when crushed which has an antimicrobial properties. Allicin is considered as the most important biologically active compound in garlic since it decomposes to other sulfur containing molecules, thiosulfonates and disulfides [7].

TABLE VIII. ZONE OF INHIBITION OF PENICILLIUM AT DIFFERENT GARLIC CONCENTRATION

GARLIC LEVEL %(w/v)	ZONE OF INHIBITION (mm)
1.5	-
2.5	4.5
3.5	7

- no inhibition



(a) (b) (c)

Figure 3. Zone of Inhibition of Penicillium at a) 3.5% b) 2.5% and 1.5% garlic level (w/v) after 3 days of incubation

IV. CONCLUSIONS

Garlic is an effective mold inhibitor for yeast-leavened bread. The optimum formulation with 3.2% (w/v) extended the shelf life of the yeast-leavened bread up to 8 days as compared to the control sample for only 4 days.

The mold isolate which was responsible for the spoilage of yeast-leavened bread was identified as *Penicillium sp*. 2.5% and 3.5% (w/v) garlic is inhibitory to the mold isolate.

It was also found out that the pH and moisture content of the yeast-leavened bread decreases during storage.

APPENDIX A. THE DIFFERENT TREATMENT RUNS OF YEAST LEAVENED BREAD

APPENDIX B. SET PLAN OF AN INCOMPLETE BLOCK DESIGN (COCHRAN AND COX 1957)

Block	I	II	III	IV	V	VI	VII
1	13	8	12	6	7	1	9
2	5	14	10	7	12	2	8
3	15	12	11	5	8	3	6
4	12	11	6	9	2	4	14
5	4	5	8	1	14	9	15
6	11	9	7	2	13	15	5
7	1	2	3	4	5	6	7
8	2	3	1	13	15	14	12
9	8	6	4	15	10	13	2
10	10	4	5	11	1	12	13
11	9	13	14	10	6	5	3
12	14	7	13	3	4	8	11
13	7	15	9	12	3	10	4
14	3	1	2	8	9	11	10
15	6	10	15	14	11	7	1

(t=15, k=7, b=15, r=4, λ=3, E=0.92, Type II)

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