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Research Article

EFFECT OF CONCENTRATION AND pH ON THE PRESERVATIVE ACTION OF CALCIUM PROPIONATE AGAINST BLACK BREAD MOLD (*RHIZOPUS STOLONIFER*) IN KERALA

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ABSTRACT
Bakery products are the important food material in various country and cultures. Breads form the major baked foods accounting for over 80% total bakery products. Bread products are easily subjected to microbial spoilage. *Rhizopus stolonifer* or black bread mold is commonly found on bread surface. The black bread mold is takes its nutrients from bread surface and cause the spoilage of bread. Preservatives are added to increase the shelf life to the bread and inhibit mold growth. Calcium propionate is an antifungal that is added to the bread product to prevent the black bread mold growth and used as a preservative in breads and other baked goods. The preservative action of calcium propionate was studied in various pH (4, 5.6 and 7) and concentrations (5 and 10%) against *Rhizopus stolonifer*. Solid media as well as liquid sabouraud media were used to access the effect of varying pH and concentration. The growth of the molds was accessed using area of growth as well as turbidity methods. Higher inhibitory action was observed on low pH and higher concentration of calcium propionate (10%). Even though preservatives increase the shelf life of food products, their impact on human health should be studied. Adjusting the pH of the food component may also prove to increase effect of food preservatives rather than increasing its concentration. More detailed investigations would be ideal to reduce the quantity of the preservatives without affecting the shelf life of products.

Keywords: *Food Preservatives; Black Bread Mold; Sabouraud Agar; Calcium Propionate*

INTRODUCTION
Bread forms one of the oldest, fermented, traditional, convenient and nutritious food of the mankind (Barkeling *et al.,* 1995; Dural and Hines, 1993; Bone, 2005; Saldeen *et al.,* 1998; Dewettinck *et al.,* 2008; Barrett, 1975; Cauvain, 2004; Lopez *et al.,* 2001). Decline in the consumption pattern of bread is seen due to the changing food habits and breakfast cereals (Dewettinck *et al.,* 2008; Siega-Riz *et al.,* 2000). New varieties of baked products with less calories, more fiber, less salts and additives are getting more attention among consumers around the globe (Dewettinck *et al.,* 2008; Meuser *et al.,* 1994). Bread product among the world vary in color, taste, texture, shape, size due to production techniques, types of flour, leavening agents and salts (Dewettinck *et al.,* 2008; Martin, 2004; Lopez *et al.,* 2001).
Microbial spoilage affect the perceived freshness of the bread and the most common microbial spoilage arise from mold growth; causes lot of economic loss in the food industry (Ryan *et al.,* 2008; Pateras, 2007; Needham *et al.,* 2005; Legan, 1993).
Alternatives to prevent microbial spoilage of bread include modified atmosphere packaging, irriadiation, paterurization and addition of propionic acid and salts (Ryan *et al.,* 2008; Legan, 1993; Pateras, 1998; Nielsen and Rios, 2000; Rodriguez *et al.,* 2008). *Rhizopus stolonifer* is a thread like mold and it is a heterotropic species, depend on sugar and starch as carbon source. It is generally found in bread or soft fruits as a food source for growth, nutrition, and reproduction (Reddy *et al.,* 1998; Bonaterra *et al.,* 2003; Bosquez-Molina *et al.,* 2010; Qing and Shiping, 2000). *Rhizopus stolonifer* consist of mycelium composed of multinucleated rapidly growing hyphae. When the spores of mold are released they produce more mycelium through germination. When the mold matures it begins to turn black. The black bread mold quickly spread over the bread surface within a few days and spores are commonly found in air. The spores grow rapidly at temperature between 15°C and 30°C (Gerez *et al.,* 2009; Legan, 1993; Hammes and Gänzle, 1997; Filtenborg *et al.,* 1996).

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Propionic acid has been widely used as a traditional chemical for bread preservation with a concentration up to 3,000 ppm (Ryan *et al.*, 2008; European Union, 2005; Ponte and Tsen, 1987). Calcium propionate is an antifungal that is added to the bread product to prevent the black bread mold growth Calcium propionate is used as a preservative in breads and other baked goods. It is used in bakery products as a mold inhibitor typically at 0.1-0.4 %. Calcium propionate is produced by propionic acid and calcium hydroxide. Calcium propionate causes health problems in humans including headache, sleeplessness, and damage of the stomach cell linings (Dengate and Ruben, 2002; Quiles *et al.*, 2007; Pattison *et al.*, 2004; Martin-Diana *et al.*, 2007; Stewart *et al.*, 1977).

Recent trends in bakery industry are to reduce chemical preservatives and minimal processing. Natural preservation includes the use of latic acid bacteria, acetic acid and plant extracts against microbial spoilage (Ryan *et al.*, 2008; Valerio *et al.*, 2008; Levine and Fellers, 1940; Brul and Coote, 1999). Black bread mold does not always result in the health problems. The average healthy person's immune system usually provides protection from harmful effects of mold. Most health problems caused by mold are from allergic reactions to it. When mold cell landed in the respiratory tract, the body's immune system response to those invading and cause allergic illness. The resulting symptoms included runny nose, scratchy throat, asthma and sneezing (Hardin *et al.*, 2003; Pepys, 1969; Fung and Hughson, 2003; Verhoeff and Burge, 1997).

Given lacking qualitative and quantitative data on effectiveness of calcium propionate as a food additive in Kerala, objective of this study were: (1) to identify and characterize importance of pH and (2) to determine the effect of varying concentration on growth of *Rhizopus stolonifer*.

MATERIALS AND METHODS

Study Area

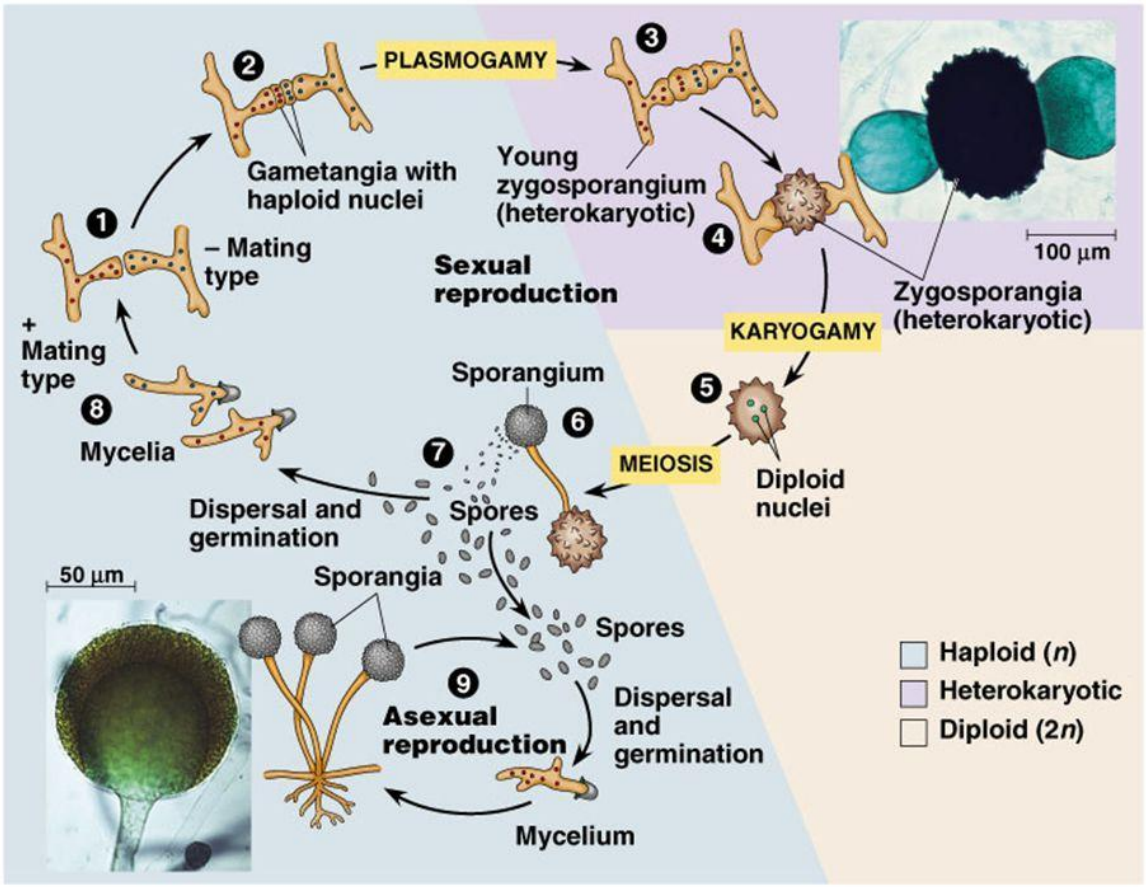

Figure 1: Reproductive cycle of *Rhizopus stolonifer* *(Adapted from Pearson Education, Inc)*

Kerala state covers an area of 38,863 km^2 with a population density of 859 per km^2 and spread across 14 districts. The climate is characterized by tropical wet and dry with average annual rainfall amounts to 2,817 ± 406 mm and mean annual temperature is 26.8°C (averages from 1871-2005; Krishnakumar *et al.*, 2009). Maximum rainfall occurs from June to September mainly due to South West Monsoon and temperatures are highest in May and November (Figure 3).

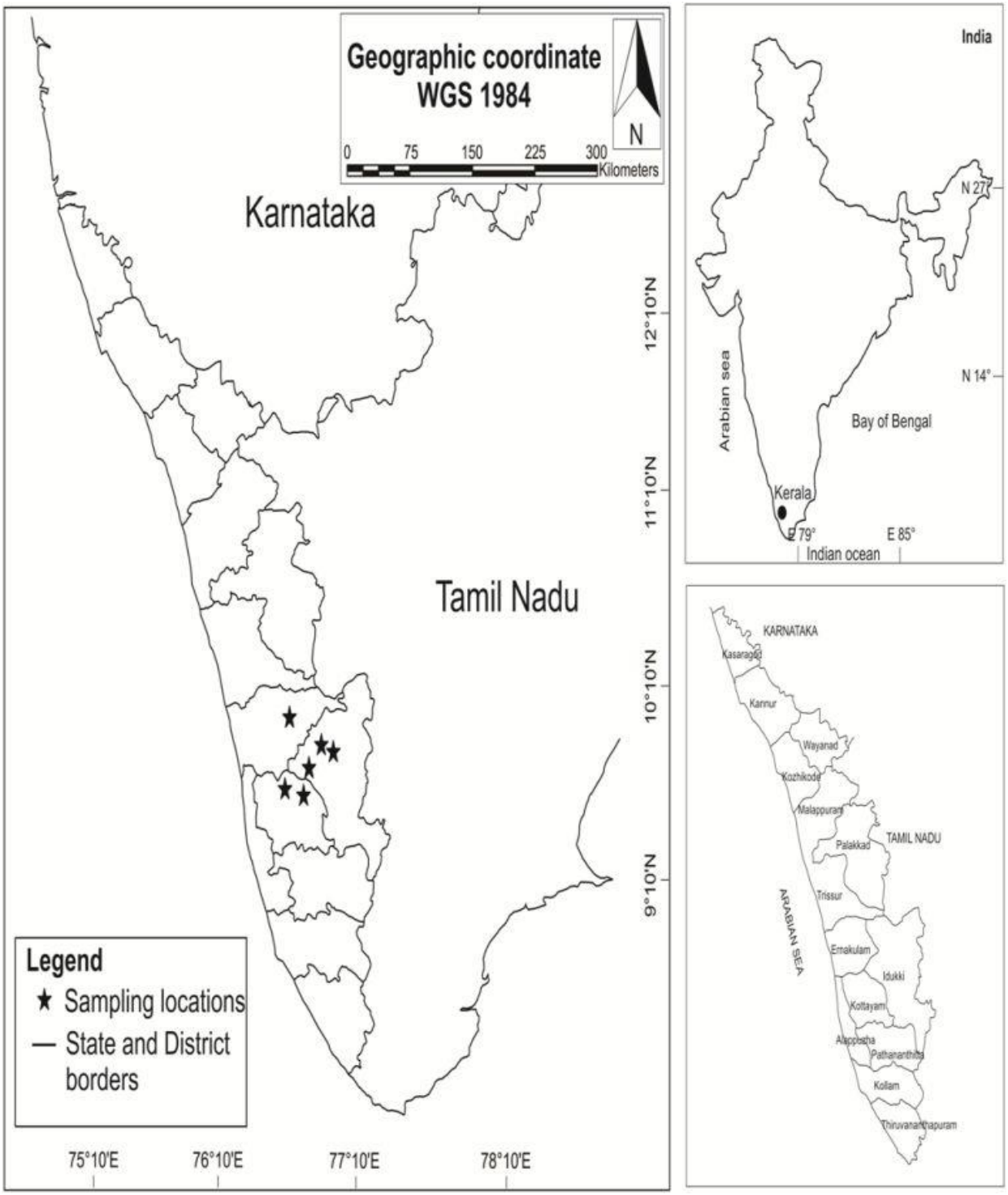

Figure 2: Map of Kerala showing the various sample collection points

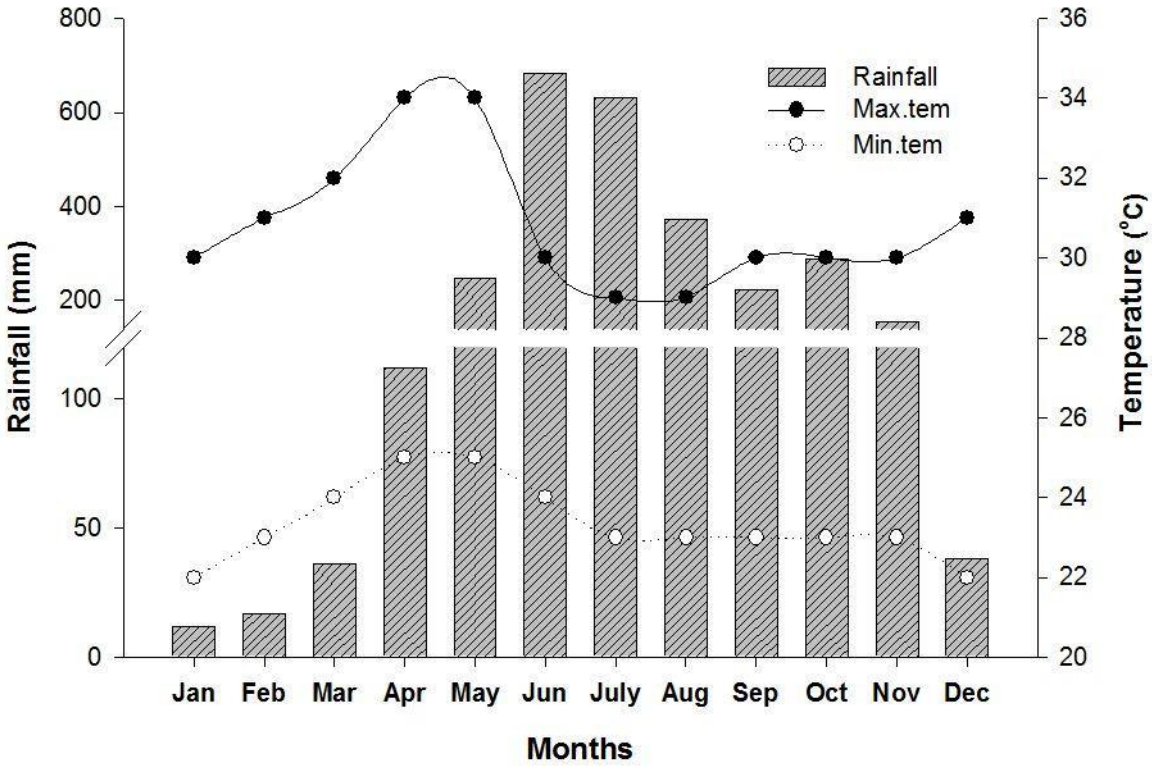

Figure 3: Mean monthly rainfall (mm), maximum and minimum temperatures (°C) in Kerala, India (1871-2005; Krishnakumar *et al.*, 2009)

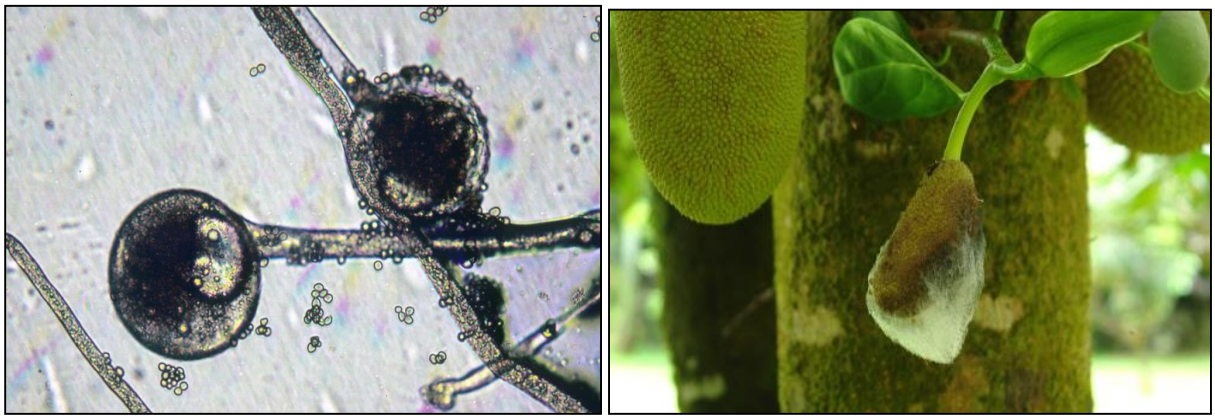

Figure 4: Preservatives, molds and breads: calcium propionate power; (top left), various types of bread; (top right), calcium propionate crystals; (middle left), *Rhizopus stolonifer* infection in bread; (middle right), microscopic view of *Rhizopus stolonifer*; (bottom left), *Rhizopus stolonifer* infection in *Artocarpus heterophyllus* fruit; (bottom right) Photo courtesy; wisegeek.com, education.com, forestryimages.org, growables.org

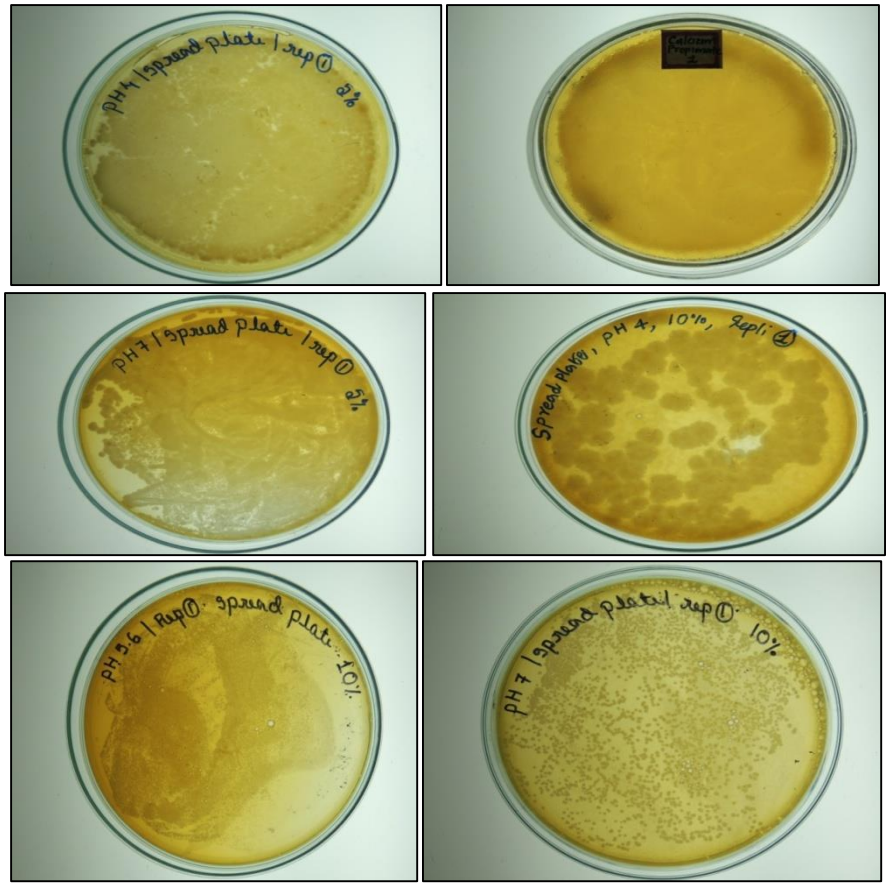

Figure 5: Action of pH (4, 5.6, 7) and concentration (5, 10%) of calcium propionate against *Rhizopus stolonifer* in sabouraud agar: pH 4 and 5% concentration; (top left), pH 5.6 and 5% concentration; (top right), pH 7 and 5% concentration; (middle left), pH 4 and 10% concentration; (middle right), pH 5.6 and 10% concentration; (bottom left), pH 7 and 10% concentration; (bottom right)

Figure 6: Action of pH (4, 5.6, 7) and concentration (5, 10%) of calcium propionate against *Rhizopus stolonifer* in sabouraud media: master plate; (top left), culture in broth; (top right), various pH at 5% calcium propionate concentration; bottom left, various pH at 10% calcium propionate concentration; (bottom right)

Sample Collection

Samples of *Rhizopus stolonifer* were collected based on an elaborative literature survey as well as traditional knowledge. A total of 6 samples were collected from different regions of the Kerala from January 2015 to February 2015. Locations of the sample collection areas were recorded using a Trimble Geoexplorer II (Trimble Navigation Ltd, Sunnyvale, California) and data were transferred using GPS Pathfinder Office software (Trimble Navigation Ltd, Sunnyvale, California).

Calcium Propionate

Calcium propionate samples were purchased from a local bread making factory. It acts as the preservative to help keep bread free of mold and increase shelf life.

Table 1: Physical and chemical properties of calcium propionate

IUPAC name	**Calcium propionate**
Molecular formula	$C_6H_{10}CaO_4$
Molar mass	186.21 g/mol
Appearance	White crystalline solids
pH value	7 – 9
Solubility	Insoluble in acetone
Crystal structure	monoclinic

Mold Growth and Isolation

Bread samples without chemical preservatives were procured from a local bakery. The slices were transferred to a pertiplates and sprayed with distilled water. The plates were kept in a moist, humid atmosphere in dark conditions. Spraying of water continued if necessary. The fungus was isolated using pure culture techniques (Lavermicocca *et al.*, 2003; Carll *et al.*, 1954; De Scott, 1965).

Identification

The molds were identified using lactophenol cotton blue staining using standard protocol (Suganthi *et al.*, 2011; Bouakline *et al.*, 2000; Farone and Farone, 2005; Kumar, 2013).

Broth Preparation and Isolation

The sabouraud broth was prepared with a pH of 5 to 6 by adding few drops of hydrochloric acid or sodium hydroxide. After the sterilization of medium inoculate the black bread mold into the broth and later incubated at 25-30 ºC for overnight (Adebayo and Kolawole, 2010; Canhoto *et al.*, 2004; Adhikari *et al.*, 2000).

Inoculation of Mold into Agar

Preparation of Agar and Addition of Calcium Propionate

Sabouraud agar media is prepared in varying pH (4, 5.6, 7) and sterilized. After sterilization of media, calcium propionate was added in two different concentrations (5 and 10%) under aseptic condition. The calcium propionate added agar were later poured into properly labeled petriplates and allowed to solidify.

Inoculation of Mold into Sabouraud Agar

Inoculation of black bread mold into sabouraud agar by spread plate technique. Using sterile L rod and micropipette mold is spread over the agar surface. Incubate the petriplate at 25-30 ºC.

Inoculation of Mold into Broth

Preparation of Broth and Addition of Calcium Propionate

Sabouraud broth was prepared in varying pH (4, 5.6, 7) and sterilized. After sterilization of media, calcium propionate was added in two different concentrations (5 and 10%) under aseptic condition using laminar air flow.

Inoculation of Mold into Sabouraud Broth

From the master plate the isolated black bread mold were inoculated into the broth using a sterile needle and incubated at 25-30 ºC for 12 hrs. After incubation the turbidity of the broths was measured using calorimeter.

Statistical Analysis

The results were analyzed and descriptive statistics were done using SPSS 12.0 (SPSS Inc., an IBM Company, Chicago, USA) and graphs were generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

RESULTS AND DISCUSSION

Mold Growth in Plates

5% calcium propionate agar plates in three different pH showed 99% of mold growth after 2 days incubation. There are no visible changes in the mold colony. 10% calcium propionate containing agar plate showed visible changes in the different three pH. In pH 4, large rounded colonies were formed. In pH 5.6, thin small colony spread over the agar while in pH 7, small rounded isolated colonies were found. This indicates the efficiency of calcium propionate is decrease with increase in pH. So mold growths were increased with increase in pH of the medium.

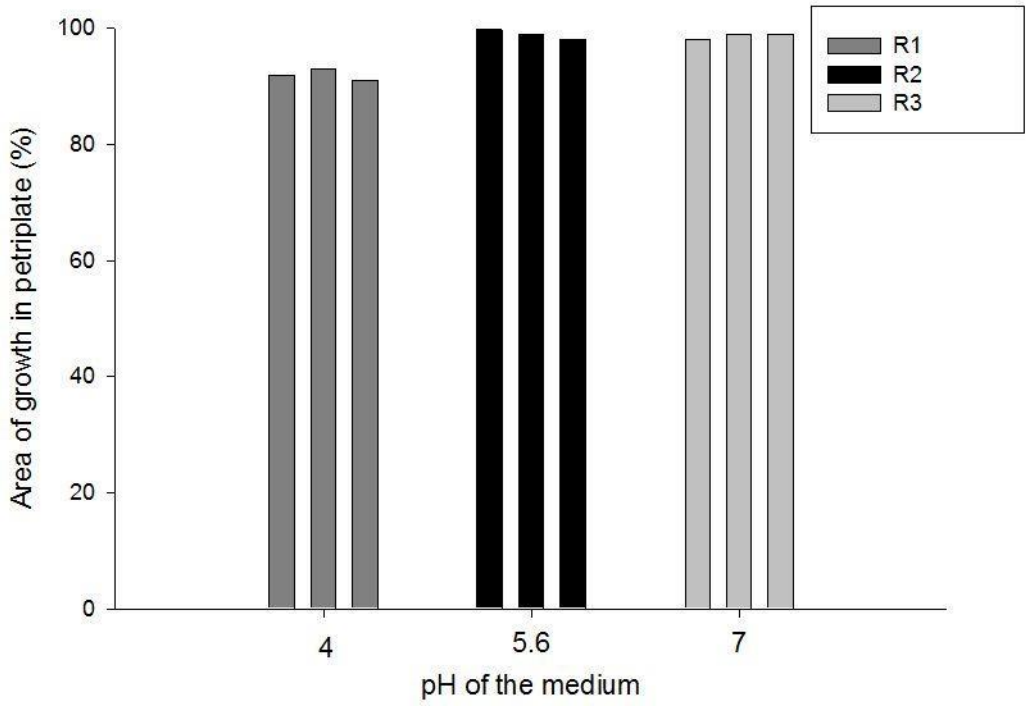

Figure 7: Growth of *Rhizopus stolonifer* on sabouraud agar plates in varying pH (4, 5.6, 7) at 5% concentration of calcium propionate. R1, R2 and R3 stands for replicate 1, 2 and 3 respectively

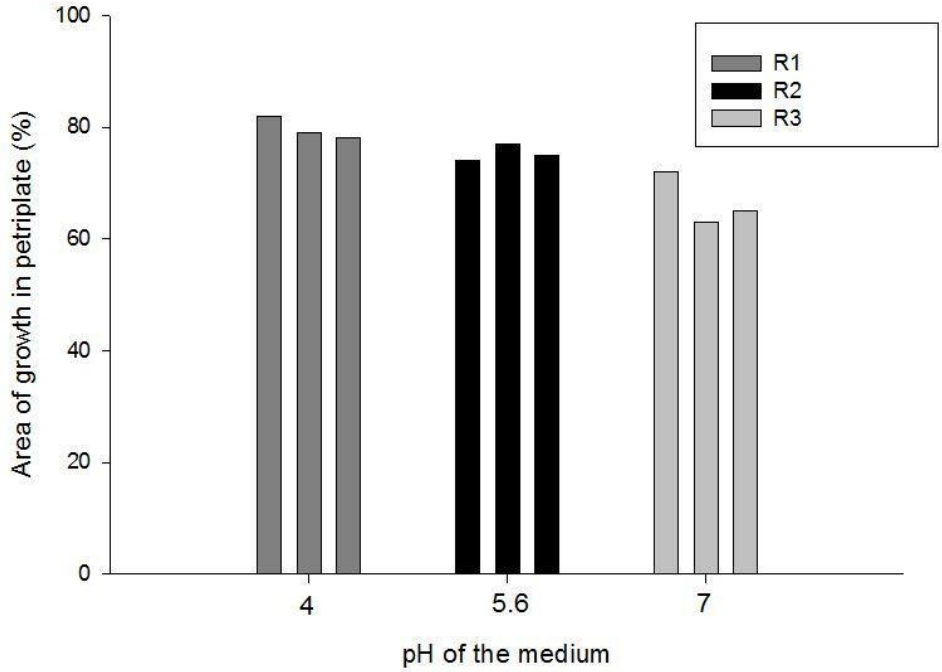

Figure 8: Growth of *Rhizopus stolonifer* on sabouraud agar plates in varying pH (4, 5.6, 7) at 10% concentration of calcium propionate. R1, R2 and R3 stands for replicate 1, 2 and 3 respectively

Mold growth in Broth

The turbidity of broth is measured by calorimeter. The optical density value is increase with increase the pH.

Table 2: Growth of *Rhizopus stolonifer* on sabouraud broth (n=3, mean ± one standard deviation) in varying pH (4, 5.6, 7) at two different concentration (5, 10%) of calcium propionate

Calcium propionate concentration (%)	pH of medium 4 Optical density	5.6	7
5	0.57 ± 0.01	0.63 ± 0.02	0.85 ± 0.02
10	0.44 ± 0.02	0.48 ± 0.01	0.65 ± 0.02

Calcium propionate is widely using food preservative in bread. Increase the pH of the food will decrease the action of calcium propionate. Here two different concentration of calcium propionate is using 5% and 10% and the comparative study of effect of calcium propionate in different pH (pH 4, pH 5.6, pH 7).
Here 5% calcium propionate containing agar plate in different three pH shows 99% of mold growth after 2 days incubation.
There is no visible change in the mold colony. But 10% calcium propionate containing agar plate shows visible changes in the different three pH. In pH 4, large rounded colony is formed. In pH 5.6, thin small colony spread over the agar. In pH 7, small rounded isolated colonies are found. This indicates the efficiency of calcium propionate is decrease with increase in pH. So mold growth is increase with increase pH.
The calcium propionate is used most extensively in the prevention of mold growth and for mold inhibition in bread. The effectiveness of calcium propionate is decrease with an increase in pH. The addition of calcium propionate is increase the shelf life of the baked product (Markus *et al.,* 2012). Calcium propionate is more active in low level pH (4.5-5.5) and water activity 0.80-0.90 (Guynot *et al.,* 2005).
Calcium propionate is produced by propionic acid and calcium hydroxide. Fungi prefer to grow at high sugar concentration and low pH. The sabouraud agar contains high concentration of dextrose and mycological peptone is an important selective medium for cultivation of fungi particularly filamentous mold.

Conclusion

Calcium propionate is added to bread products to prevent mold growth, destroying or inhibiting reproduction of black bread mold which can substantially increase shelf life of bread. The effectiveness of calcium propionate is decrease with increase in pH. So the high pH value of food material will easily subject to spoilage while food additive added. Usage of natural preservatives should be also studied to avoid the health risks of chemical preservatives.

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