



Production of bioethanol from starchy tuber (*Amorphophallus commutatus*) and antimicrobial activity study of its extracts

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Abstract

Biofuels have been regaining popularity due to the rising price of oil, along with the growing concern about global warming caused by carbon dioxide emissions. Biofuels are processed from plant resources and are mostly made up of cellulose, which is one of the toughest materials. If cellulose can be turned into biofuel, it could be more efficient than other commercially available fuels starchy tuber. The of this study is on bioethanol production from starchy tuber. The comparative study was done between biological and chemical processes for the bioethanol production using *Amorphophallus* tubers. *Amorphophallus commutatus* species was selected because it shows higher starch content as per starch estimation. Tuber was collected in the lab and pretreatment was given; followed by slurry was prepared and hydrolyzed by using fungal culture *Aspergillus* and *Trichoderma*. Three different conditions were maintained as two samples contain both fungal cultures, and one was having normal pH and temperature, and other was at normal temperature having pH 6, and third having only *Aspergillus* species and normal temp and pH. Hydrolysis was done by saccharification method. After hydrolysis sample is filtered and all three samples allows for fermentation process by using yeast (*Saccharomyces cerevisiae*) process is carried out for 12 to 15 days after fermentation the fermented sample was distilled by Soxlet Extraction method and lastly the sample was estimated for alcohol estimation by using specific gravity method. Two samples showed 11% alcohol content and third one shows 12% content when compared with alcoholometry table. Antimicrobial activity was also studied by using three extracts such as before hydrolysis, after hydrolysis and after fermentation against four types of organism's two species *Salmonella* and *S. aureus* shows positive result while *E. coli* and *Serratia sp.* showed negative result. Results indicate that chemical process more productive compared to biological process. However, biological process is eco-friendly. It is also cost-effective. It can be produced on large scale for production of bioethanol.

Keywords: Alcoholometry, Biofuel, Hydrolysis, Fermentation.

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1. Introduction

Fossil fuels are the major sources of energy, and they account for about 80% of global energy demand (Sharma et al., 2012). Since they are characterized with a lot of problems, which include non-renewability, erratic prices, global warming, ecosystem imbalance, health hazards, and other environmental or agricultural effects like pollution and food shortage. Therefore, there is need for a renewable, healthier, more environment-friendly, abundant or secure, and sustainable alternatives. Biofuels potentially provide these advantages and are increasing in global demand (Govindan et al., 2019).

Ethanol is a fuel, a solvent, an antifreeze, and an organic feed stock in the chemical industry. It can be produced chemically from petroleum source or biologically from any fermentable carbohydrate by yeast. Ethanol is a colorless liquid with the structural formula $\text{CH}_3\text{CH}_2\text{OH}$, often abbreviated as $\text{C}_2\text{H}_5\text{OH}$. Also used as the type of alcohol found in alcoholic beverages. It is mostly used as a biofuel. In common usage, it is often referred to simply as alcohol or spirits. Ethanol can be used as an alternative fuel to gasoline (Math et al., 2015).

Bioethanol can be produced by fermentation from several renewable sources, such as from potatoes and corn. (Ramaraja and Unpaprom, 2019). Globally, there is a growing interest to produce ecologically sustainable biofuels. Ethanol fermented from renewable sources for fuel or fuel additives are known as bioethanol. Additionally, the ethanol from biomass-based waste materials is considered as bioethanol. Currently, there is a growing interest for ecologically sustainable biofuels. The target in the Asian countries is to increase bioenergy contributions in total energy consumption. In Europe bioethanol is already used as additive in some gasoline products instead of toxic MTBE and TAME. (Hussain et al., 2011).

Economic factors weight heavily in choosing the method of ethanol production. When petroleum and natural gas prices are low, ethanol can be economically produced from petrochemical feed stocks; however, when petrochemicals are selling at a premium price, microbial production of ethanol from corn, molasses, or other plant material becomes more economical. When starch such as corn, and other complex carbohydrate are used as the raw material it is first necessary to hydrolyze them to simple fermentable sugars. The hydrolysis can be accomplished with enzyme from barley malt or molds or by heat treatment of acidified material. Corn, molasses, sugar beets, potatoes and grapes are some of the common raw materials employed throughout the world. Ethanol combustion produces lowered air population compared to gasoline combustion (Ramaraj et al., 2018).

The economics of ethanol production by fermentation is significantly influenced by the cost of raw material, which accounts for more than half of the production cost. Ethanol contains 35% oxygen, which results in a complete combustion of fuel and thus lowers the emission of harmful gases. Converting a renewable non-fossil carbon, such as organic wastes and biomass consisting of all growing organic matter (plants, grasses, fruit waste and algae) to fuel would assure a continual energy supply (Saha and Banerjee, 2013).

Bioethanol as an alternative source of energy has received special attention world due to depletion of fossil fuels. According to United States department of energy, for every unit of energy put towards ethanol production 1.3 units are returned (Hill et al., 2006). In India sugarcane molasses is the main raw material for ethanol production but now the short supply and increased cost is the main hindrance for its use. There are about 342 distilleries in the country with an installed capacity over three billion liters of ethanol annually (Narde, 2009).

Crops like corn, barley, wheat, rice and tubers like potato, sweet potato and suran are the major sources of starch. Potato shows 60%, sweet potato 70%, corn 56%, wheat 60% while *Amorphophallus* tuber 77.2% starch. (Kusmiyati et al., 2012). Thus, in present study suran (*Amorphophallus* sp.) is used as source for ethanol production. Araceae are family of monocotyledonous flowering plants in which flowers are borne to type of inflorescence called spadix. It is usually called Arum family. It consists of 105 genera and 3,300 species in the world.

Amorphophallus commutatus (Araceae), is a rare herb. Which was further used for study. Aqueous and organic solvent extracts of the tubers were investigated for antibacterial activity properties by using disc diffusion method. Antimicrobial activity carried out against pathogenic strains of gram-negative bacteria (*E. coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Salmonella typhi*). The different extracts differed significantly

in their antibacterial properties with the benzene extract being very effective followed by petroleum ether, chloroform and ethyl acetate extracts. Aqueous and methanol extracts (Bhuyar et al., 2019).

A. commutatus is a wild herb which belongs to the family Araceae, mainly distributed in the tropical forest regions of peninsular India. Corns of this plant have been used by traditional healers for treating various ailments. According to Jain et al. (2005) tuber of the plant has been used as antidote for snake bite. Pawra tribes of Nandurbar district of Maharashtra use the tuber paste of *A. commutatus* for curing scabies. *A. commutatus* is used by the tribal communities of Dang region of Rajasthan as cooling agent. Local communities of Kolhapur district of Maharashtra used to have tuber of this plant species for piles and the water in which tuber is boiled is used for mouth diseases. *A. commutatus* var. wayanadensis, commonly known as 'kattuchenna' is widely used among the tribal communities of Wayanad district for piles. According to the tribal healers the corns of the plant is a boon to the patients having piles. *A. commutatus* is a Red listed medicinal plant species with a vulnerable status globally. As the particular species of *Amorphophallus* is rare in occurrence and have potential medicinal values, the pockets or zones rich in its occurrence should be conserved for future. Ethnobotanical knowledge on the traditional uses of *A. commutatus* var. wayanadensis was collected through structured interviews of the Kuruma and Kurichar tribal communities of Wayanad district of Kerala, India. The efforts in our lab to evaluate pharmacological potential to this wild medicinal plant have found to be fruitful. Previous studies in our laboratory demonstrated potential antibacterial activity against pathogenic strains which validated the traditional claim of its use for microbial infections.

2. Materials and methods

2.1. Collection of plant material

The plant materials (*A. commutatus*, *Amorphophallus konkanensis*, *Amorphophallus bulbifer*, *Amorphophallus paeonifolius*) are collected from Western Ghats, Amboli and nearby places and further processed for alcohol production followed by hydrolysis.

2.2. Pretreatment of plant

Pretreatment was done by dilute sulfuric acid and distilled water. The pretreated plant was then grinded in mixer and slurry was made with sterile distilled water by 1:2 proportions. This slurry was then used for further studies.

2.3. Estimation of starch by anthrone reagent

0.5 gm of *Amorphophallus* tubers was homogenized in hot 80% ethanol to remove sugars. Residues were washed repeatedly with hot 80% ethanol. To the residues, 5 ml water and 6.5 ml 52% (HClO₄) perchloric acid was added. Centrifuged and supernatant was taken. Extraction was repeated using fresh perchloric acid. Centrifuged and the supernatant was taken and make the volume 100 ml. Pipette out 0.1- or 0.2-ml supernatant and make the volume to 1 ml by water. Prepare standard by taking 0.2, 0.4 and 1 ml of working standard make the volume 1 ml in each tube by D/W. Add 4 ml anthrone reagent to each tube. Heat for 8 min in boiling water bath. Cool rapidly and read intensity of green to dark green at 630 nm.

2.4. Estimation of reducing sugar

100 mg tuber powder was weighed, 5 ml 80% ethanol was added twice, and supernatant was collected, evaporated it by keeping in water bath at 100 °C. 10 ml water was added. Pipette out 0.5 ml of extract in test tubes and equalized the volume by 0.5 ml water. 3 ml of DNSA reagent was added later. The contents were heated in boiling water bath for 10 min. cooled and O.D. was measured at 540 nm. Similarly run the series of standard using glucose (100 mg/100 ml). Finally, graph was plotted.

2.5. Hydrolysis

Hydrolysis of starch done by using saccharification method the pretreated slurry was taken in three different flasks. Out of these, two flasks were inoculated with *Aspergillus Niger* and *Trichoderma species* and other one was only with *Aspergillus Niger*. Out of these three flasks, two flasks were kept at normal pH and other one was adjusted to pH 6 and all were incubated at room temperature. Hydrolysis was carried out for 12-14 days.

2.6. Fermentation

After saccharification process, the inoculums were added in the quantity of 1:2 concentrations in three different flasks. It includes such as one flask which is added by only yeast granules 6 gm and other two flasks were supplemented with Nitrogen source by addition of yeast extract 2.5 gm each and yeast granules 6 gm each. As

ethanol fermentation is an anaerobic process, it requires yeast *Saccharomyces cerevisiae*. So, for fermentation process, we used *Saccharomyces cerevisiae*. Initially, incubation of 24 h in aerobic conditions is favorable for the growth of yeast cells at room temperature. Then anaerobic conditions are provided to media for ethanol production.

2.7. Distillation

It was carried out in the laboratory by using soxhlet apparatus. The sample mixture was centrifuged to separate the solid debris from the solvent mixture. The solvent mixture was then loaded into a distillation flask of the soxhlet apparatus. The temperature was set to 78 °C as the boiling point of ethanol is 78 °C. Ethanol being volatile at this temperature evaporates and condenses into the inner vessel of the soxhlet apparatus.

2.8. Estimation of alcohol by specific gravity method

A specific gravity bottle is taken and thoroughly cleaned with chromic acid. The bottle is then washed with water and repeatedly washed with distilled water. The bottle is then dried by a current of hot air in the hot air oven. The stopper is also washed and dried as above. The dried specific gravity bottle is stoppered with the capillary stopper. The weight of the bottle is accurately determined using a weighing balance. The weight of the dried empty bottle is recorded as w_1 gm. Determination of weight of air free distilled water was done by filling the specific gravity bottle with air free distilled water, up to the rim. Place the capillary stopper carefully. The specific gravity bottle along water is weighted accurately. Its weight is recorded as w_2 gm. The net of air free distilled water is calculated by the difference between w_2 and w_1 , which gives the result. Determination of the weight of sample was recorded by removing the distilled water from the specific gravity bottle. The bottle was rinsed with test sample. The specific gravity bottle along the sample is weighted accurately; the weight is recorded as w_3 gm. The net of the sample is calculated by the difference between w_3 and w_1 . Determination of percentage of alcohol in the sample was carried out by the specific gravity of the sample is first calculated by the given formula from apparent specific gravity. The percentage of alcohol in test sample is determined by the ready reference table for alcoholometry (Math et al., 2015). This gives the percentage by volume of alcohol corresponding to the apparent specific gravity of sample at various temperatures.

2.9. Antimicrobial activity

Agar plates were spread by four different bacterial cultures (*E. coli*; *Salmonella*; *S.aureus*; and *Serratia sp.*). Three wells were prepared by cork borer in which three different extracts were added 100 μ l; after the incubation of 24 h zone of inhibition was observed.

3. Results and discussion

3.1. Estimation of starch and sugar

Starch estimation from above four samples of *Amorphophallus* species was done by using anthrone reagent. As shown in Figure 1 *Amorphophallus commutatus* showed highest starch concentration that was 0.19 mg/ml, so it is further used for ethanol fermentation.

Sugar and starch estimation is done by DNSA and anthrone method respectively. Result observed which showed that before hydrolyses starch concentration was 75 μ g/ml, i.e.; but after hydrolyses it decreased to 51 μ g/ml as shown in Figure 2. As per opposite direction sugar concentration was increased up to 0.63 mg/ml after hydrolyses to the concentration which was observed before hydrolyses 0.36 mg/ml as shown in Figure 3.

3.2. Antimicrobial Activity

Simultaneously antimicrobial activity was carried using three different extract such as before hydrolysis, after hydrolysis and after fermentation extracts using four different pathogens such as *E.coli*; *Salmonella*; *S.aureus*; *Serratia* and result observed (Figure 4 A and 4B) were that *Salmonella* and *S. aureus* showed positive result while *E. coli* and *Serratia sp.* showed negative result as shown in Table 1.

3.3. Fermentation

Three different sample conditions were maintained as follows sample 1 was maintained to pH 6.0 and supplemented with Nitrogen source. Sample 2 was maintained to normal temperature and pH also supplemented with Nitrogen source. Sample 3 was not supplemented with nitrogen source. After hydrolyses fermentation was performed for alcohol production and alcohol concentration was estimated by specific gravity method. Result observed were sample 1 and 2 supplemented Nitrogen source are having 11% alcohol concentration and 12% concentration which is having without Nitrogen source respectively as shown in Figure 5.

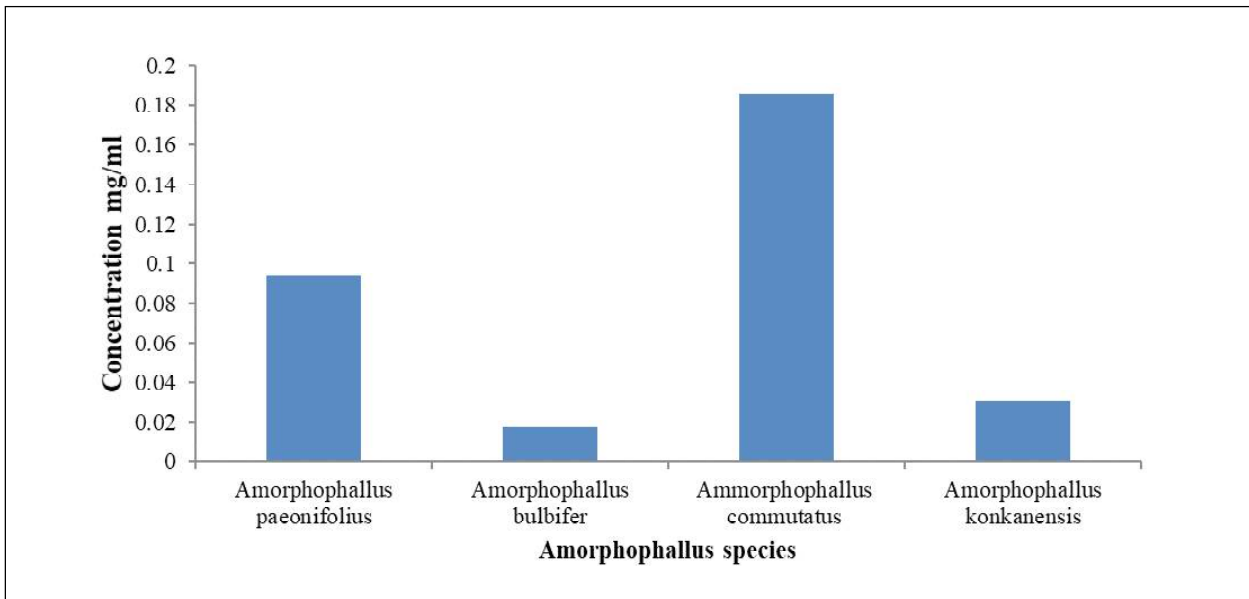


Figure 1: Estimation of starch in *Amorphophallus* species

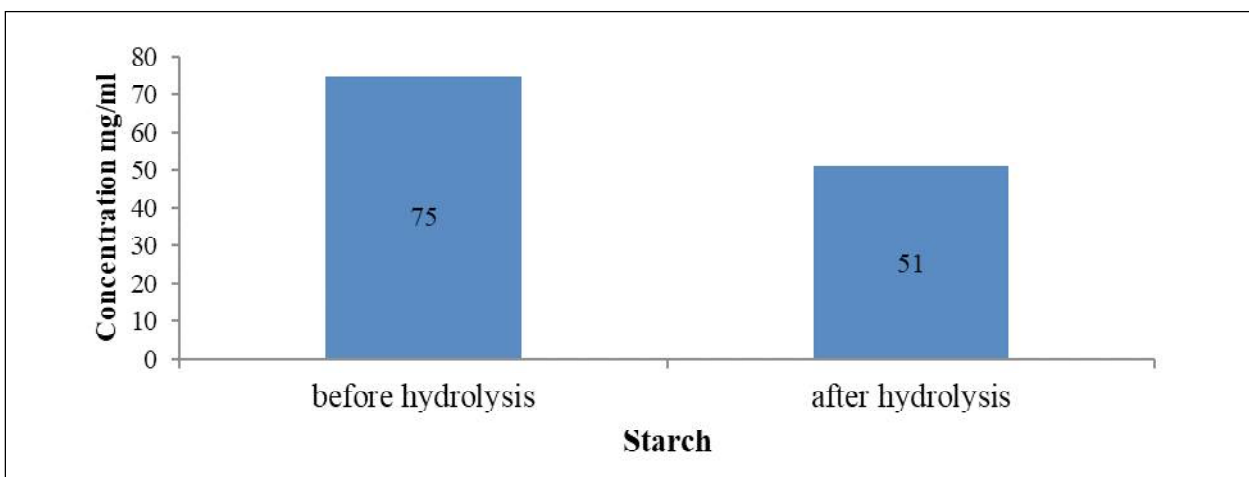


Figure 2: *Amorphophallus commutatus* tuber

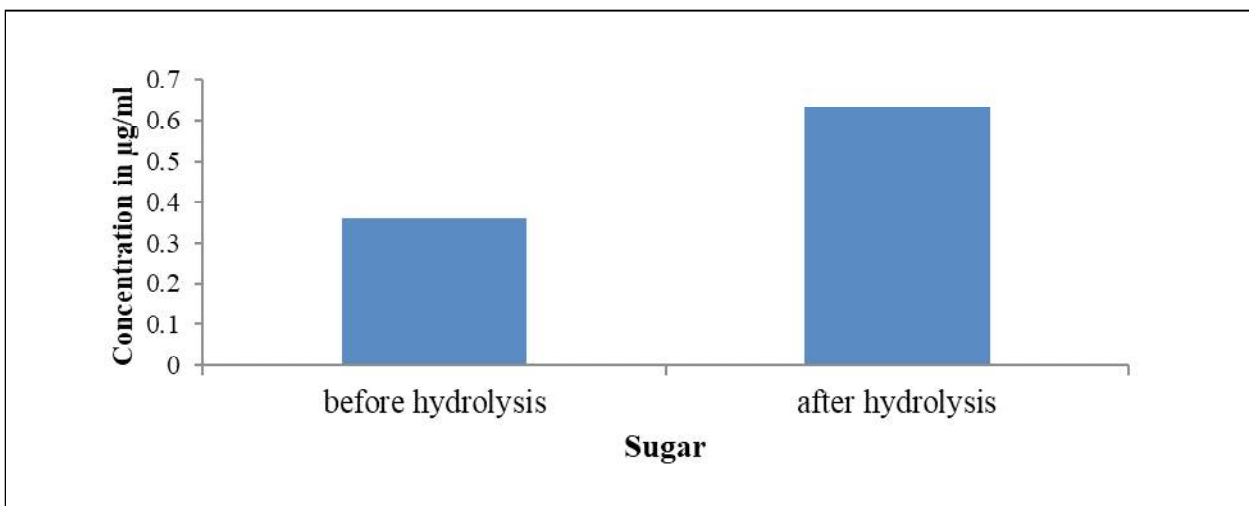
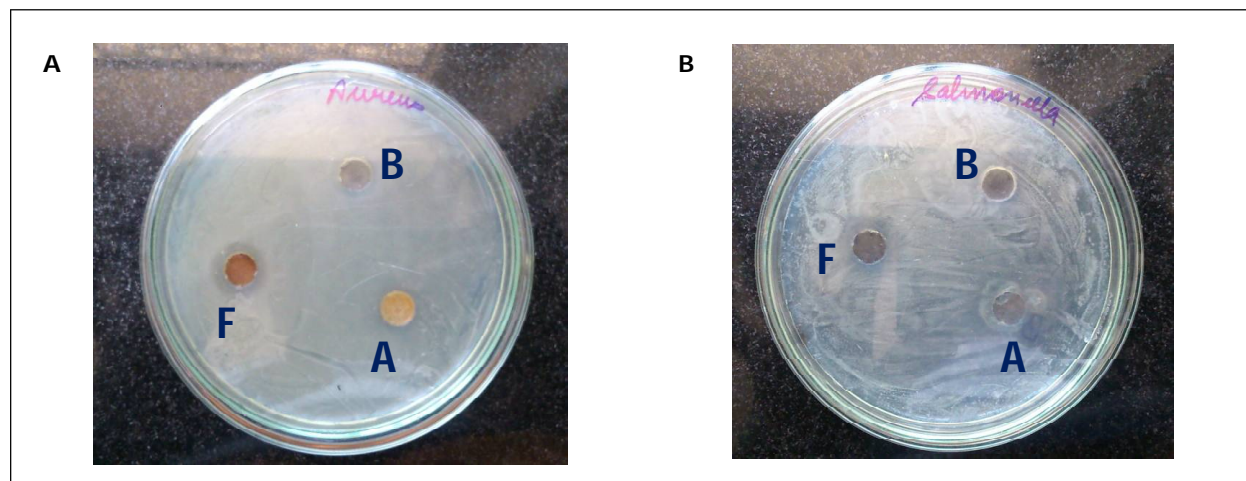


Figure 3: Estimation of sugar in *Amorphophallus commutatus* tuber



Note: Mean ± SE with similar superscripts (a, b) in the same row differ significantly at $P < 0.001$. Significant at $***P < 0.001$. Figures in parentheses represent number of cells observed in each case.

Figure 4A: Antimicrobial activity of tuber extracts against *S.aureus* Figure - 4B: Antimicrobial activity of tuber against *Salmonella*

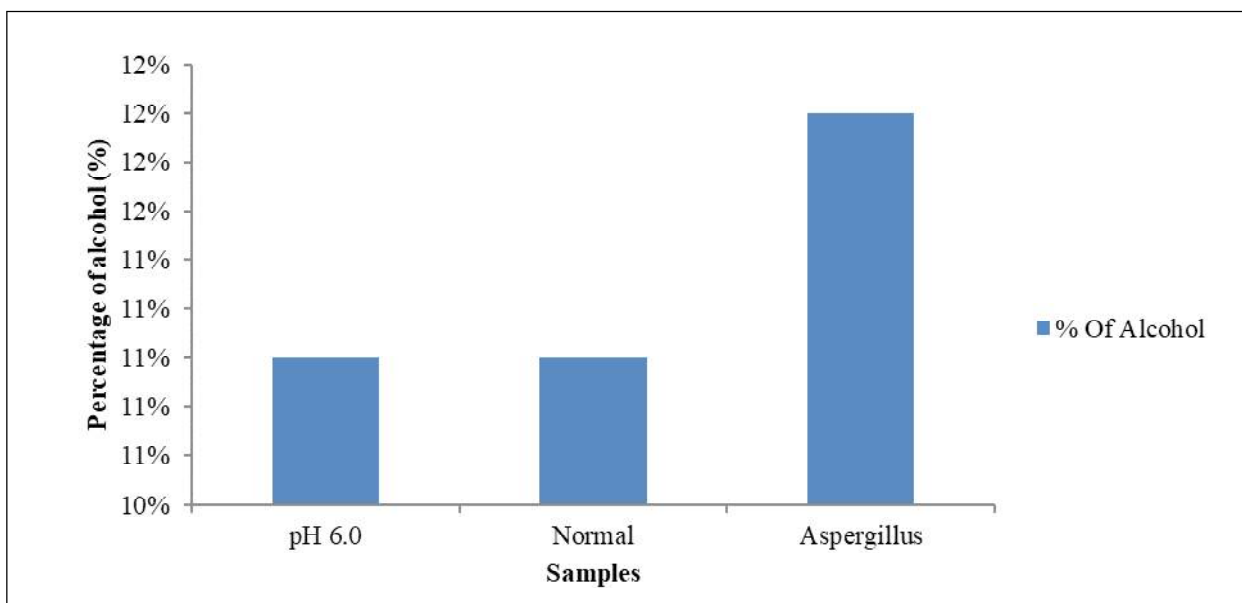


Figure 5: Percentage of alcohol produced from *Amorphophallus commutatus* after fermentation

Sample	Activity
1. <i>E.coli</i>	-
2. <i>Salmonella</i>	++
3. <i>S. aureus</i>	+
4. <i>Serratia</i>	-

Note: '-' No Activity; '+' Low Activity; and '++' Medium Activity.

Calculation:
 $W_1 = 24.340, W_2 = 51.350, W_3 = 50.830/50.840/50.820$
 Weight of water ($W_2 - W_1$) = 26.49/26.50/26.48
 Weight of sample ($W_3 - W_2$) = 27.01/27.01/27.01
 Specific gravity of sample = (Weight of sample × Density of water in RT)/Weight of water
 $(W_3 - W_1) \times 0.9944 / (W_2 - W_1) = 0.9752/0.9752/0.9748$
 11% alcohol estimated in Samples 1 and 2; and 12% alcohol in Sample 3 were taken from International alcoholometry table.

4. Conclusion

Amorphophallus commutatus was selected for fermentation due to higher starch content as per starch estimation. Tuber was collected in the lab pretreatment was given; slurry was prepared and hydrolyzed by using fungal culture *Aspergillus* and *Trichoderma*; Three conditions were maintained two samples contain both fungal cultures and one was having normal pH and temperature and other was at normal temperature having pH 6 and third having only *Aspergillus* species and normal temp and pH. Hydrolysis was done by saccharification method. After hydrolysis sample is filtered and all three samples allows for fermentation process by using *Saccharomyces cerevisiae* process is carried out for 12 to 15 days after fermentation the sample was distilled by Soxhlet Extraction method and lastly the sample was estimated for alcohol estimation by specific activity method. Two samples got 11% alcohol content and third one shows 12% content. Antimicrobial activity was also studied by using three extracts such as before hydrolysis, after hydrolysis and after fermentation against four types of organisms two species *Salmonella* and *S. aureus* shows positive result while *E.coli* and *Serratia sp.* showed negative result.

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