Ministry of Education and Science of Ukraine

ODESSA NATIONAL ACADEMY OF FOOD TECHNOLOGIES

International Competition of
Student Scientific Works

BLACK SEA SCIENCE 2021

PROCEEDINGS

ODESSA, ONAFT 2021

Ministry of Education and Science of Ukraine

Odessa National Academy of Food Technologies

International Competition of Student Scientific Works

BLACK SEA SCIENCE 2021

Proceedings

Odessa, ONAFT 2021

UDC 001.8(063)

Recommended for print by the Academic Council of
Odessa National Academy of Food Technologies
on April 6, 2021, Protocol No. 13

Black Sea Science 2021: Proceedings of the International Competition of Student Scientific Works / Odessa National Academy of Food Technologies; B. Iegorov, M. Mardar (editors-in-chief.) [*et al.*]. – Odessa: ONAFT, 2021. – 731 p.

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1. FOOD SCIENCE AND TECHNOLOGIES

EFFICIENCY OF WINE AND BIOETHANOL PRODUCTION BY THE YEAST (PICHIA KUDRIAVZEVI) OF TODDY AND BAKER'S YEAST (SACCHAROMYCES CEREVISIAE)

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Abstract. *The toddy was collected from local area Mamsapuarm Srivilliputhur. The sample was serially diluted and plated for screening of yeast. The isolate was subjected to cultural, morphological, biochemical and molecular characters. It was subjected to sugar fermentation and ethanol tolerance test was performed. The identified organism named Pichia kudriavzevi (Yeast). The yeast fermented glucose galactose, sucrose, xylose, maltose, raffinose, fructose, arabinose, lactose and the ethanol for 0, 2.5, 5, and 7.5 were performed. It was found to be 7.5% of ethanol contains 9.4x10^5 (CFU/ml). The colour change was observed in bushnellhass broth from blue to dark brown which indicating the degradation of hydrocarbon. The isolated yeast was about 0.30 ± 0.02 mg/ml and purchased yeast was 0.26 ± 0.02 mg/ml. The yeast content of the isolated yeast wine was 10.7 × 10^{5}and purchased yeast wine was 6.1 × 10^5.The bacterial content in isolated yeast wine was 3.2 × 10^{5}and purchased yeast wine was 3.8× 10^5.The antioxidant activity of the isolated yeast wine was 3.05 ± 0.0 µg/ml and purchased yeast wine was 3.05 ± 0.0 µg/ml. The total acidity of the isolated yeast wine was 0.31 ± 0.08 and purchased yeast wine was 2.00 ± 1.0 µg/ml. The protein content of the isolated yeast wine was about 2.6 ± 0.08 µg/ml and purchased yeast wine was 2.2 ± 0.08 µg/ml. Out of three fruit peels, pine apple shows the 1.940 ± 0.00U/ml. From the agricultural waste, corn stalk produced amount of ethanol was 2.31 ±0.00 U/ml. Potato exhibits the amount of ethanol was 2.10 ± 0.00 U/ml. Sugarcane possess increased amount of starch was 0.09 ± 0.01 mg/ml. Out of the substrates, potato was produced the amount of enzyme 0.653 ±0.00 mg/ml.*

Key words: Yeast. Wine, Ethanol, Hydrocarbon degradation, Agricultural waste

I. INTRODUCTION

Toddy is a customary alcoholic fermented beverage produced from sap of trees from the family *Palmea*. It has been planned that the fermentation process starts immediately after the sap has been collected; naturally occurring microorganisms even with the collecting container, ferment the available carbohydrate. The process is accelerated by increase in temperature. Fermentation time varies from a few hours to a few days, depending on the time of the day and climatic conditions when the sap is collected several yeast and bacterial species including homo and hetero-fermentative lactic acid bacteria, the alcohol producing bacteria [1, 2].

Wine is cheap and represents an important part of income in rural areas and it's the most popular alcoholic beverages consumed by socio–economic strata society in Tamil Nadu. Unfortunately this wine produced is not stable, the fermentation process continues until the quality becomes unacceptable. Everyday huge quantities are poured away. Palm wine is consumed to alcohol for its nutritional effect because of its probiotic content. Palm wine is a beverage produced by fermentation of sugars present in sap of palm trees by the yeast and bacteria have by all means been exposed to varying concentration of ethanol and acids. The strains that survive to any extent in that wine must have some degree of ethanol tolerance, which is a monumental importance in choosing a yeast strain for industrial ethanol fermentation. Yeasts are widely dispersed in the environment. It grow best in neutral or slightly acidic pH environment. Some are normal flora on skin surfaces, others are parasitic or symbiotic and are mostly common in environments where there is a sugar-rich material. Some are associated with soil and insects reported that palm wine is a suspension of different types of microorganisms including bacteria, filamentous fungi and yeasts.

Yeasts occur in palm wine as indigenous micro flora and are mainly from the genus *Saccharomyces, Schizo saccharomyces, Pichia, Candida, Saccharomycoides*. Several studies have reported on the capabilities of different yeasts to utilize hydrocarbons. The cumulative demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives has necessitated increased production of this alcohol. Ethanol production is usually talented by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products. Owing to depleting reserves and challenging industrial needs of petrochemical feed stocks, there is global emphasis in ethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology [3]

Therefore, yeasts, lactic acid bacteria and acetic acid bacteria are considered to play the most important role in the palm wine production .Worldwide, the two attributes are exploited industrially, and suitable strains of yeast are used in production of beers, wines, breads, fuel alcohol, nutritional yeast biomass and by-products from these. Yeasts usually used for baking are carefully selected strains of *Saccharomyces cerevisiae [4]*. An irresistible wealth of information on genetics, molecular biology and physiology has accumulated on this organism making this traditional species the best-characterized eukaryotic system today. Several limitations, in terms of using *S. cerevisiae* as a tool for heterologous production, have been reported in the past. These included reduced biomass yield due to aerobic alcohol fermentation, very low yields (with a maximum of 1-5% of total protein), hyper glycosylation, plasmid instability, and the retention of protein in the periplasmic space. It is due to these limitations that

attention was redirected to non-*Saccharomyces* or non- conventional yeasts as hosts for heterologous production of proteins However, with the developments in the field of fermentation, different strains of *Saccharomyces cerevisiae* and various fruits juices have been explored for wine production [5].

The present work is aimed to screen the beneficiary yeast from toddy and their possible performance of microorganisms for ethanol, wine production and hydrocarbon degradation etc.

2. Material and methods

2.1. Isolation and identification of yeast

Fresh palm wine samples from palmaya palm (*Borrassus flabellifer*) were collected from traditional palm wine trapper from Mamsapuram in Virudhunagar District, Tamilnadu, India. One ml of the palm wine sample was collected aseptically and serially diluted in sterile peptone water and 0.1ml aliquots of suitable dilution was inoculated in duplicates by spread plate method on Sabouraud Dextrose Agar (SDA) for Yeast. The inoculated plates were incubated aseptically 24-48 hours for the yeast at 22^0C. The yeast was morphological identified by using microscope then the cultured and maintained on SDA agar medium. After optimum growth the culture was stored at refrigerator for further use [6].

2.2. 18S r RNA gene sequencing and Phylogenetic analysis

PCR on the extracted DNA was performed on 100 Volume. Oligonucleotide primers with specificity for yeast 16S rRNA genes, primers 18S rDF (CGCTGGCGGCAGGCTTAACA); 18S rDR (CCAGCCGCAGGTTCCCCT) were used to amplify the 18S rRNA gene fragments with template DNA.

2.3. Sugar fermentation

The test described by was carried out using 1.0g each of glucose, lactose, fructose, galactose and sucrose [7].

2.4. Ethanol tolerance test

The yeast isolate was tested for ethanol tolerance.

2.5.Estimation of ethanol

The alcohol content of sample was estimated by potassium dichromate method [8].

2.6. Hydrocarbon degradation by using selected organisms

One milliliter of palm wine was inoculated into 200 ml sterilized mineral salts broth as described by Mills. Crude oil samples were filtered and autoclaved at 121°C for 15 mins for sterility and when cooled added to the inoculums and swirled for proper mixing at concentration of 1% (v/v). Incubation was done for fourteen days at room temperature (25 ± 2°C) without shaking. The spread plate technique was used to

inoculate the viable cultural isolates. The un-adapted microorganisms were obtained by inoculating 0.1 ml of the fresh palm wine onto sterile mineral salt agar plates in triplicates using the spread plate method [7-8].

2.7. Screening for biodegradation potentials

A loopful of 48 hour culture of the isolates was inoculated into a sterile 100 ml of Bushnell Haas broth containing 1 ml of sterile crude oil (1% v/v) and 1 ml of redox indicator (2% v/v of 2, 6-dichlorophenol indophenols). A control was also set up containing no microorganisms. The set up was left for 15 days [9].

2.8. Microbial Analysis

Microbial population of wine was evaluated using SDA (yeast), Nutrient Agar (bacteria), spread plate technique one ml of wine sample is serially diluted and spread the agar plates and plates were incubated in 37ºC in 24 hrs for bacteria 48 hrsfor yeast. After incubation the colonies were count in CFU/ml.

2.9. Ethanol estimation on winesEthanol estimation on wines was carried by [10]

2.10. Determination of total antioxidant activity on wines

Total antioxidant activity on wines [11]

2.11. Estimation of total acidity on wines

The total acidity was calculated by using following formula [11]

2.12. Estimation of Protein on wines

The amount of protein was calculated by [12] Lowry *et al* (1951).

2.7. Agro substrates

Agro industrial wastes such as pineapple, papaya, pomegranate peel, Sugar cane waste and vegetables waste were used for ethanol production was carried by [13]

2.7.1. Preparation of growth media in fruit peels

Pineapple papaya, pomegranate obtained from local market sivakasi were washed, and their outer coats i.e. removed, cut in small pieces using a knife and then blended with sterile distilled water in ration of 1:10 respectively using electric blender and stored in refrigerator prior to use. Peptone, 0.1%,Malt extract, 0.1% (w/v), Yeast extract, 0.2% (w/v), Calcium carbonate 0.2% (w/v); Ammonium phosphate, 0.2% (w/v), Ferrous sulphate 7H2O, 0.001% (w/v) respectively. *Saccharomyces cerevisiae* growth medium was prepared using yeast peptone broth at pH 5.5 [13]

2.7.2. Preparation of Corn Stalks and sugarcane

The corn stalk obtained are from matured stalk that have been harvested for the corn. After obtaining the corn stalk, the leaves were removed and then dried at the temperature of 60-70°C until constant weight was achieved. The dried corn stalk then were chopped manually into small pieces of 10-30 mm. Finally the corn stalk were grinded into smaller pieces of 0.2 [14]

2.7.3. Preparation of growth medium in waste vegetables

2 ml ready for the enzyme.,100% of molasses as control,80% of sugarcane + 20% of yeast extract, 70% of sugarcane + 20% of yeast extract + 10% cauliflower water., 70% of sugarcane + 20% of yeast extract + 10% cabbage water, 70% of molasses + 20% of yeast extract + 10% cauliflower water + 10% cabbage water.

2.7.5. Preparation of growth medium in potato

In order to attain maximum fermentable sugar conversion optimum parameters are applied. Temperature: 30 °C pH: 6 and Incubation time 84 hrs. Ethanol is mostly produced depends on quality of raw materials and the optimum conditions. Alcohol from potatoes obtained from two enzymatic methods with yeast only. Using Alpha amylase and yeast. First of all raw potatoes of market are being washed properly as contamination along with layers could be removed. After that mash of potatoes were prepared as smooth as silk with the help of grinder or juicer by adding proper amount of distilled water. Homogeneously of solution is made which is after wards processed to bio chemical or enzymatic reaction [15]

2.8. Reducing sugar estimation

Reducing sugars were analyzed by dinitrosalicylic acid (DNS) method [16]

2.9. Starch estimation

The starch content of sample analyzed [17]

2.10. Enzyme assay for ethanol production (Assay of amylase)

The amount of amylase activity was determined by [17]

3.0. RESULTS

3.1. Isolation of **yeast** from toddy

Yeast was isolated from toddy in sabouraud dextrose agar. (Fig.1 and 1a).

Fig.1 Yeast colonies isolated from toddy in SDA agar plates

3.1a. 18s RNA Gene sequencing

TAAAGATTAAGCCATGCATGTCTAAGTATAAGCATTATACGGTGAAACTGCGAA
TGGCTCATTAAATCATTTATCGTTTATTTGATAGTTCCGTTCTACATGGATAACCGTGGA
AAATCTAGAGCTAATACATGCGTAAAGCCCCGACTTCGGGAGGGGTGTATTTATTAGA
TAAAAAATCAATGCCCTCGGGCCTTTTGATGATTCATAATAACTTTTCGAAGCTCATGG
CCTTGCGCCGGAGCTGGTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATA
GAGGCCTACCATGGTTTTCACGGGTAACGGGGAATAAGGGTTCGATTCCGGAGAGGGA
GCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC
CTGACACAGGGAGGTAGTGACAATATATAACGATACAGGGCCTTTGGTCTTGTAATTG
GAATGAGTACAATGTAAATACCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCA
GCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGC
TCGTAGTTGAACTTTGGGCCTGGGCGGACGGTCTACCTATGGTAAGCACTGTTGCGGCC
GGGTCTTTCCTTCTGGCTAGCCCTCGGGCGAACCAGGACGATTACTTTGAGGAAATTAG
AGTGTTCAAAGCAGGCCTTTGCTCGGATATATTAGCATGGAATAATAGAATAGGACGC
ATGGTTCTATTTTGTTGGTTTCTAGGACCATCGTAATGATTAATA

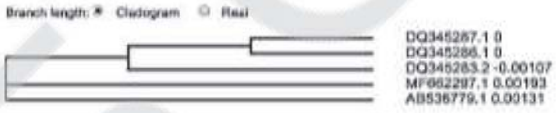

Fig. 1a. 18s RNA Gene sequencing of selected strain (*Pichia kudriavzevi*)

3.2. Wine production from isolated yeast and commercial yeast

By the substrate the wine produced and filtered by using Whatman No.1fiter paper. The wine was maintained at suitable condition (Fig.1b)

(a) (b)

Fig .1b. Wine production from isolated yeast and commercial Yeast

3.3. Microbial analysis

Yeast count

Both are spread in YPG agar plates the 107x10^5 CFU/ml colonies was observed (Table 1 and 2).

Table.1. Yeast count for wines (Both)

Wines	CFU/ml
Isolated yeast wine	10.7×10^5
Commercially purched yeast	6.1 ×10^5

3.4. Bacterial count

Both are spread in nutrient agar plates the 30 colonies was observed in isolated yeast wine plate and 58 colonies were observed in commercially purchased yeast wine plate and calculated by CFU/ml(Table 2).

Table 2. Bacterial count for wines (Both)

Type of wines	CFU /ml
Isolated yeast wine	3.2×10^5
Commercially purched yeast	5.8 ×10^5

3.5. Ethanol estimation of collected toddy sample at different time interval

In first day onwards, the maximum activity of ethanol was 0.992mg/ml at 0.5 followed by 1.0 ml of toddy sample. It was gradually increased 1.0mg/ml at 0.5 and 1.0ml concentrations of ethanol (Fig.2.).

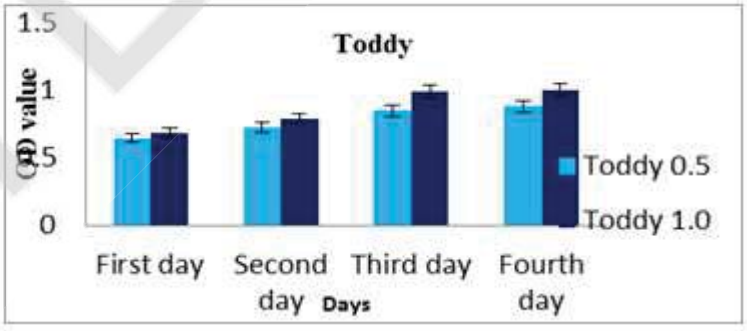

Fig.2.Estimation of toddy for ethanol production at various time interval (Days)

3.6. Ethanol estimation of wines

The amount of ethanol was estimated for the isolated yeast wine and commercially purchased yeast wine. The estimation was compared. Thus isolated yeast wine was 0.30 ± 0.12 mg/ml. The commercial yeast wine was 0.26 ± 0.02 mg/ml (**Fig.3)**.

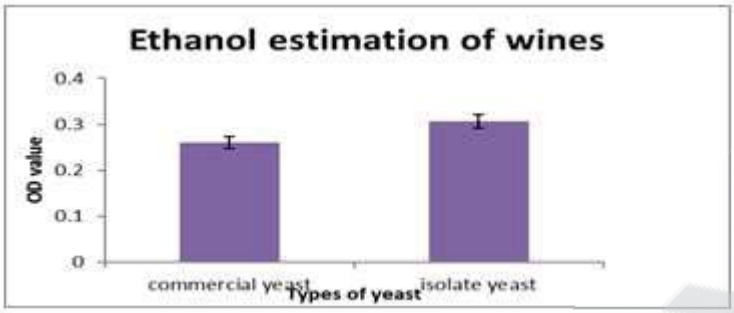

Fig. 3. Estimation of ethanol of wines.

3.7. Sugar fermentation test for isolated yeast

The isolated yeast cells were further subjected to carbohydrate fermentation test for identification the sugar fermentable. The isolated yeast having positive for glucose, galactose, sucrose, maltose, raffinose and fructose (Table 3).

Table 3. Sugar fermentation test

Sugars	Fermentation by yeast
Glucose	Positive
Galactose	Positive
Sucrose	Positive
Xylose	Negative
Maltose	Positive
Raffinose	Positive
Fructose	Positive
Arabinose	Negative
Lactose	Negative

3.8. The Ethanol tolerance test

The ethanol tolerance test was done for isolated yeast. Various % of concentrations like 0, 2.5, 5, and 7.5 were performed. It was found to be 7.5% of ethanol contains $9.4x10^5$ (CFU/ml) (Table.4)

Table.4. Ethanol tolerance test for isolated yeast

Ethanol concentration (%)	Yeast (CFU/ml)
0	18.9×10^5
2.5	15.2×10^5
5	11.9×10^5
7.5	9.4×10^5

3.9. Hydrocarbon degradation using enrichment of organism and Spread plate of the culture from broth

The collected palm wine was further enriched in mineral salt broth. The enriched wine was spreader in the plate of mineral salt agar. This was said to unadopted colonies. Because of the colony morphology of the plate was observed on the next day onwards (Fig. 4 and 5)

Fig.4 and 5.Enrichment of palm wine with mineral salt broth Spread plate of palm wine of adopted and unadopted organism in toddy. Adapted: Enrichment the organisms; Unadapted: Non enriched organisms

3.10. Screening for biodegradation potentials

The degradation of the crude oil (Diesel, Petrol and Kerosene) was performed. (Fig.6 and 7).

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Fig. 6. Colour change was observed in Bushnell hass broth which indicating the degradation of crude oil by yeast at 7^{th} day **A** -Control (broth+ dye+crude oil) **B**-Test-(broth+ dye+crude oil+isolated organisim)

Fig.7. Colour change was observed in Bushnell hass broth which indicating the

degradation of crude oil by yeast at 14th day **A -** Control (broth+ dye+crude oil) **B-** Test-(broth+ dye+crude oil+isolated organisim).

3.11. **Anti-oxidant property of wine samples**

The antioxidant property of the isolated wine and commercially purchased wine was analyzed. Thus the isolated yeast wine was (3.04 ±0.08) µg/ml and it was fine to scavenge the free radicals. Thus commercially purchased yeast was (3.01± 0.08) µg/ml of the antioxidant activity (Fig 8).

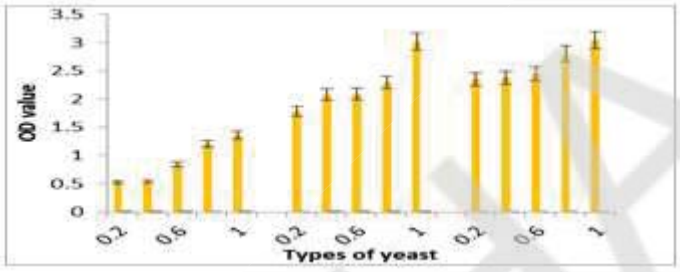


Fig.8. Estimation of total antioxidant activity of wine

3.12. Total acidity of wines

The total acidity of isolated wine and the commercially purchased wine was observed. Thus, the commercially purchased wine sample shows 0.31± 0.08 µg/ml. The isolated yeast showed 2 ± 1.07 µg/ml of the acidity content (Fig .9).

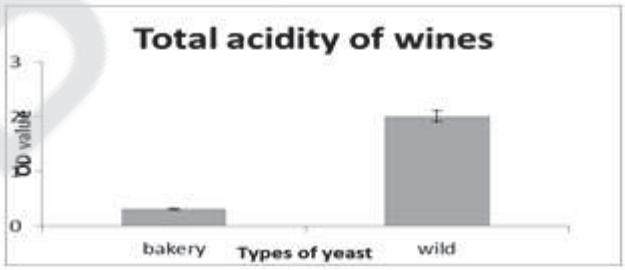


Fig.9. Total acidity of wine in isolated and commercial yeast

3.13. Estimation of Protein wines

The protein content in the wine was estimated. The isolated yeast wine and the commercially purchased wine sample were analyzed for the protein content. The

protein content in the isolated yeast wine showed about 2.6 ± 0.08 µg/ml. The commercially purchased wine showed 2.2 ±0.081µg/ml (Fig.9a)

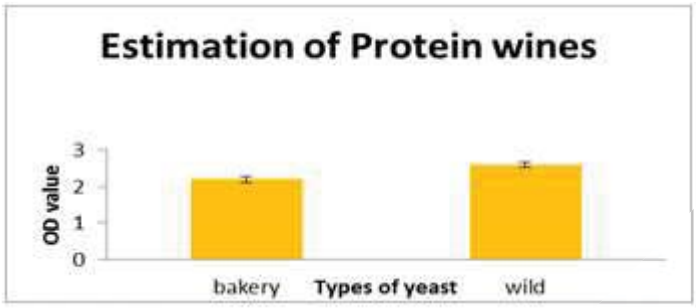


Fig.9a. Estimation of Protein wines

3.14. Fermentation of different fruit peel

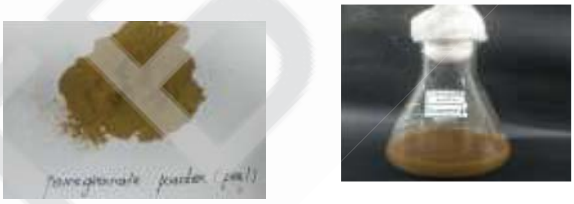
After the pretreatment the substrates were subjected to ethanol fermentation. (Fig. 10a, b, c, d, e f).

3.15. Fermentation of sugarcane and corns

The sugarcane bagasse were rich in cellulose content (Fig11a, b and 12a,b).

3.16. Fermentation of vegetable waste

The potato waste contain complex sugars which on saccarification breakdown into simple sugars, which were used by yeast for their metabolic process. (Fig. 13a and b, 14a, b, c).



(a) (b)

Fig.10.

a) Pomegranate substrate (b) Medium of pomegranate peel substrate.

(c) (d)

Fig10 c) Pineapple peel substrate 10**(d)** medium of pineapple peel substrate

(e) (f)

Fig.10 (e) Papaya peel substrate (f) medium of papaya peel substrate

(a) (b)

Fig.11 (a) sugarcane substrate **(b)** fermentation medium of sugarcane substrate

(a) (b)

Fig.12 (a) corn substrate (b) fermentation medium of corn substrate.

(a) (b)

Fig.13. (a) Potato substrate (b) Fermentation medium of potato substrate.

(a) (b) (c)

Fig14. (a) Cabbage substrate (b) Cauliflower substrate (c) Fermentation medium of vegetable waste. Fermentation of the medium takes place within 7 days .the substrate act as a carbon source for the growth of yeast. The enzyme added break down the complex sugar into simple form that can be utilized by yeast.

3.21. Enzyme estimation

Out of these potato have increased enzyme value was 0.6 U/ml± 0.02 and the minimal enzyme value was observed in corn at 0.074 ± 0.17 U/ml (Fig.19)

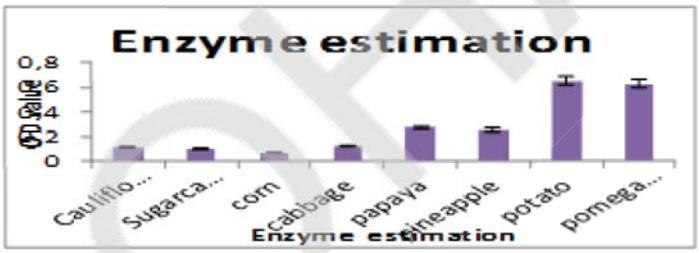


Fig.19 Enzyme estimation

5. Conclusion

The present was mainly intensive on the successful production of wine and bioethanol using varying agricultural wastes with cheap manner with respect to the selected strain. Furthur the selected strain was confirmed as superior strain with above said investigation.

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