ISOLATION OF YEAST STRAINS AND SCREENING FOR AMYLOLYTIC, PECTINOLYTIC ACTIVITIES AND PRODUCTION OF ALCOHOL FROM DIFFERENT FRUIT AND VEGETABLE WASTES

BY

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CERTIFICATE

Mr. P. VIJAYA BHASKAR REDDY, has satisfactorily prosecuted the course of research and that the thesis entitled **"ISOLATION OF YEAST STRAINS AND SCREENING FOR AMYLOLYTIC, PECTINOLYTIC ACTIVITIES AND PRODUCTION OF ALCOHOL FROM DIFFERENT FRUIT AND VEGETABLE WASTES"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

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DECLARATION

I, **P. VIJAYA BHASKAR REDDY**, hereby declare that the thesis entitled **"ISOLATION OF YEAST STRAINS AND SCREENING FOR AMYLOLYTIC, PECTINOLYTIC ACTIVITIES AND PRODUCTION OF ALCOHOL FROM DIFFERENT FRUIT AND VEGETABLE WASTES"** submitted to the Acharya N.G. Ranga Agricultural University for the degree of **MASTER OF SCIENCE IN FOOD SCIENCE AND TECHNOLOGY** is a result of original research work done by me. It is further declared that the thesis or part thereof has not been published earlier in any manner.

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Place: Hyderabad

C O N T E N T S

CERTIFICATE

This is to certify that the thesis entitled **“ISOLATION OF YEAST STRAINS AND SCREENING FOR AMYLOLYTIC, PECTINOLYTIC ACTIVITIES AND PRODUCTION OF ALCOHOL FROM DIFFERENT FRUIT AND VEGETABLE WASTES”** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN FOOD SCIENCE AND TECHNOLOGY** of the Acharya N.G. Ranga Agricultural University, Hyderabad is a record of the bonafide research work carried out by **Mr. P. VIJAYA BHASKAR REDDY** under our guidance and supervision. The subject of the thesis has been approved by the Students Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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LIST OF ABBREVIATIONS

%	:	Per cent
CD	:	Critical difference
CMIE	:	Centre for Monitoring Indian Economy
DAHP	:	Di-ammonium hydrogen orthophosphate
et al.	:	and others
Fig.	:	figure
g	:	grams
g/g	:	gram per gram
g/L	:	gram per litre
h	:	hours
ha	:	hectare
i.e.,	:	which is to say, in other words
IMTECH	:	Institute of Microbial Technology
kg	:	Kilogram
KMS	:	Potassium metabisulphate
L	:	litre
mg	:	milligram
mg/L	:	milligram per litre
mL	:	milli litre
MTCC	:	Microbial Type Culture Collection
NCIM	:	National Collection of Industrial Microorganisms
NCL	:	National Chemical Laboratories
NS	:	Non significant
^oB	:	Degree brix
^oC	:	Centigrade (or) celsius
OD	:	Optical density
pH	:	Negative logarithm of hydrogen ion activity
ppm	:	Parts per million
SEm	:	Standard error of mean
TSS	:	Total soluble solids
v/v	:	Volume per volume
vol	:	volume
w/v	:	Weight per volume
w/w	:	weight per weight
Y1	:	yeast culture 1
Y2	:	yeast culture 2

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ABSTRACT

The present investigation was carried out with the objective of isolating pectinolytic and or amylolytic yeast cultures and fermentative production of alcohol from over ripened mango and tomato pulp. Seven samples were obtained from different places and isolates were purified which were identified as yeast cultures based on morphological, cultural and biochemical characters. Some of the isolates AM-113, AM-115 and AM-117 had slight pectinolytic activity and none of the isolates had amylolytic activity. All the isolates had alcohol producing ability but highest alcohol production (80.00 g /L) was noticed with *S. cerevisiae* MTCC 172 and lowest was with isolate AM-111 (54.00 g/L).

Mango and tomato pulp was subjected to treatments with pectinase and amylase to increase the flowability of the pulp and also to increase the availability of

sugars etc for utilization by yeast culture for conversion to alcohol. The addition of pectinase to mango and tomato pulp did not show increase in alcohol content. Treatment of mango pulp with amylase had considerable increase in alcohol production where as treatment of tomato pulp with amylase did not show improvement. Treating the mango pulp with both enzymes did not show increase in alcohol whereas in tomato considerable increase was observed. The alcohol production was very low when substrate alone is used in both mango and tomato pulp. To observe whether simple addition of sugar also would help in increasing alcohol production compared to enzyme treatments, sugar was supplemented to pulp before initiating fermentation. The alcohol content nearly doubled when sugar is added to the substrate in both mango and tomato pulp compared to substrate alone, but alcohol content was lower in both cases when compared to enzymes treated pulp.

Based on the present study it is established that over ripened mangoes and tomatoes can be used effectively for fermentative production of alcohol using pre-treatment with enzymes.

CHAPTER – I

INTRODUCTION

India is the second largest producer of fruits and vegetables after Brazil and China. Mango (*Mangifera indica*) and tomato (*Lycopersicum esculentum*) are one of the important fruit and vegetable crops in India. The total area under mango cultivation is 1.6 million ha with a total production of 10.78 million tonnes (CMIE, 2004) and total area under tomato cultivation is 54 million ha with a total production of 7.65 million tonnes (CMIE, 2004). Due to lack of post harvest facilities 30 – 40% of the fruits and vegetables are being wasted every year which accounts to around 6750 crore rupees.

Being perishable in nature, the fruits and vegetables require a quick disposal and in a period of glut much of the produce is being wasted. Generally mango can be processed into the products like juices, jams, toffee, bars, milk shakes etc. Tomato can be processed into different products such as purees, ketchups, sauces etc. Good ripe fruits and vegetables are necessary and it requires specific variety for the above purposes. One of the profitable approaches is to convert over ripened and damaged fruits for production of ethanol one of the biofuels by fermentation. Some research work was carried out in India in regards to use of damaged fruits like guava and banana for ethanol production (Bhat *et al*. 1987).

Ethanol is one of the largest organic chemicals produced in large quantities by fermentation process all over the world. The demand for ethanol is increasing in recent years, because of its wide use in chemical and potable industries, medicine and as a motor fuel. It is used as a feedstock for the production of various chemicals, including acetaldehyde, acetic acid, ethyl acetate, ethyl chloride, etc. It is also used in antifreeze compounds and rocket fuels. Its use as a motor fuel is comparatively of recent origin, which has virtually replaced the petroleum in countries like Brazil. Anhydrous ethanol can be added to petroleum upto 20% (v/v) without modification to vehicle engines. Modified engines in Brazil run entirely on ethanol and in this case 95% (v/v) ethanol can be used (Hacking, 1986). With current regulations on the composition of gasoline in areas where air pollution has been a problem, fuel ethanol has taken on its most valued role as an oxygenated gasoline additive (Anon, 1996). The energy of a gallon of ethanol is greater than the total energy of all fossil fuels consumed in ethanol production (Shapouri *et al*., 1995).

Countries such as Brazil and USA started manufacturing large quantities of ethanol and used it as motor fuel not only as such (96%) but also as gashol (10-20% ethanol in gasoline). The ethanol production by these two countries amounts to nearly 40% of the total world production. Brazil tops the list when compared to per capita consumption of 24 L in USA and 1.1 L in a developing country like India. India uses this alcohol in the ratio of 40 : 60 for potable and industrial purposes. There are 285 distilleries in the country producing about 2.7 billion litres of ethanol annually (Rekha *et al*., 1997), but still ethanol production is in deficit.

Ethanol is commercially produced either by chemical synthesis (or) by fermentation. Chemical synthesis (Thermo chemical) is achieved from ethylene by esterification hydrolysis, or by direct hydration, which requires higher temperature and pressure. A complex mixture of products are formed by chemical synthesis. However, due to economic reasons ethanol synthesis from ethylene is almost abandoned and is being exclusively produced in recent years by fermentation method, utilizing the raw materials like black strap molasses, using microorganisms, operating at low temperature and producing few byproducts.

In India the alcohol production is directly dependent on the availability of molasses; the output varies according to the annual production of sugarcane. The price of sugarcane molasses has been hiked several folds, due to the decontrolling of molasses. Thus utilization of molasses for the production of ethanol is becoming not very economical for the industries. One tonne of sugar produce gives 190 L of molasses (50-55% sugar) and 280 L of ethanol can be produced per ton of molasses. (Chaudhari and Chincholkar 1996)

The present investigation is carried out to produce alcohol from the substrates like over ripened mango and tomato. Yeast cultures are screened for amylolytic and pectinolytic activities. Yeast with these enzyme activities would utilize the carbohydrates and pectins present in mango and tomato and convert them to simple sugars by which the yeast can utilize those sugars and produce more alcohol. This may fetch some price to mango and tomato farmers and while reducing environmental pollution and also the fermented material can be used as a source of organic matter reducing pressure on fossil fuels.

Objectives

1. Isolation and screening of yeast strains for amylolytic, pectinolytic activity and alcohol production.

2. Feasibility of using different substrates like over ripened tomato and mango / waste from mango processing industry for production of alcohol.

CHAPTER – II

REVIEW OF LITERATURE

2.1 ISOLATION AND SELECTION OF YEASTS FROM DIFFERENT SOURCES

As early as 1836, sugar fermentation was ascribed to vital activity of yeast by Cagniand – Latour and the fungal nature of the yeast was recognized by Schwann (1837). At this time bottom fermentation yeast was used only in Baviria, but its use spread rapidly in Europe and then to US, because of emigration of many German brew masters. Later Pasteur, in 1860 reported that different organisms affected different fermentations, and this work was subsequently extended by Hansen, 1888 (Kleyh and James Hough, 1971).

Yeasts were isolated from horticultural sites, molasses and local areas by Adams (1964), Pastoriza *et al.* (1967) and Sikovec (1976) for wine and beverage production, adding to existing strains of yeasts isolated earlier for this industry.

Seventeen strains of yeast were isolated from coconut palm wine of Srilanka by Jayatissa *et al.* (1978) of which the predominant species were *Saccharomyces cerevisiae* and *Saccharomyces exigus*. The very first report on the isolation of *Kluyveromyces apiculata, Saccharomyces chevalieri, Torulopsis* spp., *Rhodotorula rubra* and *Cryptococcus albidus* from grapes was made in India by Ethiraj *et al.* (1979). In 1980 survey of yeast flora of coconut palm wine and nipa palm wine was made in Philippines by Yamagata *et al.* (1980) and *Saccharomyces chevalieri* was shown to be dominant species associated with the palm sap.

Yeast strains were also selected for the production of alcohol from various other substrates such as lactose, inulin, starch and molasses (Moulin and Galzy, 1981; Guiraud *et al*., 1981 b; Frelot *et al*., 1982; Shan and Dahiya, 1983).

Screening of strains for efficient production of alcohol was carried out from the yeasts obtained from type cultures, breweries and alcohol factories (Tabera *et al*., 1985 and Kuriyama *et al*., 1985).

Phaff (1986) says that most species used in these various processes such as alcoholic beverages, biomass (baller's Food & Fodder yeasts) and various metabolic products, were obtained only as chance isolates and were subsequently modified and selected for better performance by elimination of undesirable characters. A strain of *Saccharomyces cerevisiae* X18, was isolated and characterized for ethanol production from palmyrah palm sap by Jeyaseelan and Seevaratham (1986), who found that the strain showed a rapid rate of fermentation and also produced much higher quantities of protein and biomass than the earlier used Y7 strain.

Studies for continuous alcohol fermentation using *Saccharomyces cerevisiae* was made by Borzani and Aquarone (1957) and Feichter and Ettlinger (1964). Reports are available on the better yield of ethanol, using cell vaccum and cell recycle methods, as the product (ethanol) is removed which is toxic to the organism (Cysewski and wilke, 1977 and Goma and Minier, 1982).

Duvunjak and Kosaric (1981) cited that *Saccharomyces cerevisiae* is the predominant species which was used to carryout large scale fermentation of ethanol. The authors studied the production of alcohol by *Saccharomyces diastaticus* and compared it with *S. cerevisiae* 125 and found that *S. diastaticus* showed lesser sensitivity to inhibition by ethanol and also had 93% efficiency in a 17% glucose medium.

Six distillery yeasts and one laboratory haploid strain of *Saccharomyces cerevisiae* were screened for their invertase level and amount of ethanol production in sucrose medium. It was found that the level of this enzyme in the distillery yeast is high, variable and tolerant to both high temperature as well as alcohol concentrations encountered in Indian distilleries. The level of this enzyme however did not correlate with the rate or amount of ethanol production by these yeasts (Babita Gupta and Tauro, 1995).

2.2 ISOLATION OF AMYLOLYTIC AND PECTINOLYTIC YEASTS

The yeast *Saccharomyces cerevisiae*, the main organism used for alcoholic fermentation on an industrial scale, lacks amylolytic and pectinolytic activity and is unable to utilize starch and pectins during the vegetative phase. Since the use of starch and pectins as renewable biological resource has both socio and economic advantages.

The extension of the substrate range of *S. cerevisiae* by the recruitment of heterologous sequences specifying amylolytic enzymes has been the subject of several studies. In most of these constructs, heterologus α-amylase (or) glucoamylase genes from various organisms have been expressed and excreted. Co-expression of the *Bacillus amyloliquifaciens* α-amylase and the *S. diastaticus* gluco-amylase genus in recombinant *S. cerevisiae* resulted in the production of higher amylolytic enzymes (Steyn and Pretorious, 1990).

Both gene cloning and protoplast fusion were used to transfer DNA from *Saccharomyces diastaticus* to the bakers' yeast *Saccharomyces cerevisiae*. The glucoamylase gene of *S. diastaticus* has been successfully cloned into *S. cerevisiae* (Anwar Nasim *et al.*, 1991).

A recombinant strain of *Saccharomyces cerevisiae* was constructed that contained the genes encoding a bacterial alpha-amylase (Amy1), a yeast glucoamylase (STa2) and a bacterial pullulanase (PulA). The *Bacillus amyloliquifaciens* alpha-amylase and *Saccharomyces cerevisiae* var diastaticus gluco-amylase genes were expressed in *S. cerevisiae* using their native promoters and the encoded enzymes secreted under direction of their native leader sequences. Introduction of PUL 1 into a *Saccharomyces cerevisiae* strain containing both STA 2 and Amy 1, resulted in 99% assimilation of starch (Janse and Pretorious, 1995).

Demores *et al.* (1995) constructed *Saccharomyces cervisiae* strain that is capable of utilizing starch. These strains were examined by Birol *et al.* (1998) for ethanol production, growth characteristics, starch degradation, enzyme extraction in batch cultures.

Lin *et al.* (1998) constructed an amylolytic yeast by multiple integration of the *Aurobasidium awamori* glucoamylase gene into *Saccharomyces cerevisiae* chromosome.

Strauss *et al.* (2001) investigated that what type of enzymes are being produced by non-Saccharomyces yeast isolated from grapes in South Africa wine yards and grape juice. In this study, 245 yeast belonging to the genera *Kloeckera, Candida, Debaromyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyeromyces* were screened for the production of extra cellular pectinases, proteases, beta glucanases, lichenases, beta glucosidases, cellulases, xylanases, amylases and sulfite reductase activity. This study clearly revealed that potential of non-Saccharomyces wine yeasts to produce a wide range of useful extra cellular enzymes during the initial phase of wine fermentation.

A total of 15 strains were isolated from natural sources including fruits, soil, molasses, honey and variety of indigenous fermented foods. Screening of these strains for growth, ethanol production and gluco-amylase activity led to selection of a yeast strain SM-10 identified as *Saccharomyces diastaticus* having maximum glucoamylase activity (80 units/ml) and ethanol production from starch (3.5%). Ethanol production from wheat flour was found to be 1.75%, which could be increased to 5.2% after treatment of flour with pepsin, diastase and gluco-amylase (Sharma *et al*., 2002).

2.3 ALCOHOL PRODUCTION FROM DIFFERENT SUBSTRATES

As early as 1947 Rogasa *et al*. produced ethyl alcohol from whey and suggested that whey was a good source of medium for ethyl alcohol production and also Adams (1949) said that different cannery wastes are used for production of alcohol by using microorganisms.

Ten mango varieties were used for alcohol beverage production i.e. wine making and concluded that wine made from varieties Fazri, Langra and Chausa were found to be good and sweet wine made from variety Dashehari was also adjudged as good for its characteristic fruity flavour (Kulakarni *et al*., 1980).

Sugarcane juice was used for production of ethanol by Rolz and Carbera (1980). A novel process for fermentation of starch was also outlined, which was characterized by omission of starch cooking step, carried out in the presence of α-amylase and gluco amylase (Lytzen, 1981; Ueda and Koba, 1981).

Ethyl alcohol was produced from apple pomace with a montrachet strain of *Saccharomyces cerevisiae* is described. More than 43 g of ethyl alcohol could be produced per kg of apple pomace at 30^{o}C in 24 h. The fermentation efficiency of this process was approximately 89% (Hang *et al*., 1981). This was reviewed by Ghose and Bisaria (1981) that different substrates and methods adopted influence the concentration of ethanol produced.

Subhash Chand and Gopalakrishnan (1983) used abundantly available Mahua (*Madhuca latifolia*) flowers in the north-eastern and central India, for ethanol production and obtained a yield coefficient of 0.49.

Neelima and Tauro (1983) used sugar beet which grows in North India during winter with high yields and high sugar content and showed that about 8.6% ethanol could be produced within 24 h. Ramaswamy and Oblisami (1983) *Zymomonas* and seven (7) strains of *Saccharomyces* isolated for the conversion of sweet sorghum and showed that the yeast varied widely in sugar consumption, biomass production and ethanol production. Toyana *et al*. (1984), produced ethanol from sweet potato tubers by applying enzyme preparations. The potential use of soybean seed coat for commercial production of alcohol by various yeast strains was reported by Srivastava (1984). Maize heated at low temperature was also used to carry out industrial alcoholic fermentation by Matsumoto (1985). Fikret *et al*. (1985) have produced a maximum of nearly 9% (w/v) of alcohol from sweet sorghum by solid state fermentation using *Saccharomyces cerevisiae* NRRL-11572.

Banana peels were used for ethanol fermentation by Tewari *et al*. (1986) after saccharification of the banana peels by acid enzyme and steam.

Jerusalem artichoke was also used for ethanol production using the organisms with inulinase enzyme (Chabbert *et al*., 1985 and Bajpai and Margaritis, 1986).

Direct fermentation of unhydrolyzed potato starch to ethanol by monocultures of an amylolytic fungus, *Aspergillus niger*, and cocultures of *A. niger* and *Saccharomyces cerevisiae* was investigated. Amylolytic activity, rate and amount of starch utilization, and ethanol yields increased several fold in coculture versus the monoculture due to the synergistic metabolic interactions between the species. Optimal ethanol yields were obtained in the pH range 5 to 6 and amylolytic activity was obtained in the pH range 5 to 8. Ethanol yields were maximal when fermentation were conducted an aerobically. Increasing *S. cerevisiae* inoculum in the co-culture from 4 to 12% gave a dramatic increase in the rate of ethanol production, and ethanol yields of > 96% of the theoretical maximum were obtained within 2 days of fermentation. These results indicate that simultaneous fermentation of starch to ethanol can be conducted efficiently by using co-cultures of the amylolytic fermenter, *Saccharomyces cerevisiae* Mohamed Abouzied and Adinarayana Reddy (1986).

Candida krusei was isolated from damaged guava and banana and was compared with *Saccharomyces cerevisiae* CDRI and *Saccharomyces cerevisiae* NCIM 3095 at different pH, nitrogen and phosphate levels for ethanol production from mixed fruit juice. *Candida krusei* was found to have same ethanol yield as *Saccharomyces cerevisiae* CDRI NTG but required 20 h more fermentation of mixed juice as compared to CDRI NTG strain (Bhat *et al*., 1987).

Mohd. Abouzied and Reddy (1987) in a study to evaluate a single-step process for enhanced fermentation of unhydrolysed potato starch to ethanol using synergistic cultures of an amylolytic yeast (*Saccharomyces fibuligera*) and *Saccharomyces cerevisiae* (non-amylolytic). The authors reported that the efficiency of conversion of starch was 90% and the co-culture helped significantly in improving the process economy.

Zymomonas mobilis strain ATCC 31822 was compared with *Saccharomyces* sp. PDR80 (a highly productive distillery yeast) for the fermentation of sugarcane molasses diluted to 17^0 Brix (12% sugars). In 3 experiments, the two organisms gave similar final ethanol concentrations (5.6 – 6.1% and 5.8 – 6.0% respectively), but fermentation was slower with *Zymomonas mobilis* than with *Saccharomyces* sp. (Murphy 1988).

Concentrations of volatiles during alcoholic fermentation of diluted Brazilian molasses (170 g $litre^{-1}$) by *Saccharomyces cerevisiae* were monitored by a gas chromatograph. Effect of various additives, notably carbohydrates and alumina beads were studied at 0.2% w/v. Ethanol concentration after 10h was 35 g $litre^{-1}$ with no additives, 52 g $litre^{-1}$ with chitin, 57 g $litre^{-1}$ with alumina (or) yeast extract and 46 g $litre^{-1}$ with chitin + yeast extract; values after 24 h (when fermentation ceased) were 61, 82, 75, 77 and 71 g $litre^{-1}$, respectively. These higher ethanol concentrations were accompanied by lower acetaldehyde concentrations and higher concentration of fusel alcohols. Such stimulation of fermentation was not observed with beet molasses media (total sugars 110 g $litre^{-1}$). The stimulation by chitin (or) alumina was probably due to adsorption of inhibitors; chitin is recommended for accelerating bioethanol production (Cachot and Pons, 1991).

Fusarium oxysporum F_3 alone (or) in mixed culture with *Saccharomyces cerevisiae*- 2541 fermented soluble and insoluble carbohydrates of sweet sorghum stalk directly to ethanol. Both microorganisms were first grown aerobically and fermented sorghum stalk to ethanol thereafter. During fermentation insoluble carbohydrates were hydrolysed to soluble sugars by the cellulolytic systems of *F. oxysporum*. Ethanol yields as high as 24.4 and 33.5 g 100 g^{-1} of dry stalks were obtained by *F. oxysporum* and the mixed culture respectively, representing a theoretical yield enhancement of 11.6 and 53.6% respectively. The corresponding ethanol concentration in the fermentation medium were 4.6 and 6.4% (w/v). These results clearly demonstrated that a large portion of insoluble carbohydrates from sorghum was converted by simultaneous saccharification and fermentation to ethanol, making the process promising for bioethanol production (Lezinou *et al*., 1995).

Sweet sorghum carbohydrates were simultaneously saccharified and fermented to ethanol by a mixed culture of *Fusarium oxysporum* and *Saccharomyces cerevisiae* in a bioreactor. *F. oxysporum* was grown aerobically for the production of the enzymes necessary for the saccharification of sorghum cellulose and hemi-cellulose. *Saccharomyces cerevisiae*, together with *F. oxysporum*, converted the soluble sugar to ethanol. Three batches of sorghum were used, harvested at different periods of the year. The optimum yield of bioconversion and ethanol concentration was 5.2 – 8.4 g of ethanol / 100 g of fresh sorghum and 3.5 – 4.9% (w/v) respectively, depending on the composition of sorghum stalks. In an experiment, the ethanol yield exceeded the theoretical, based on soluble sugars by 20.0 – 32.1% due to bioconversion of polysaccharides to ethanol (Mamma *et al*., 1996).

The effect of pre-treatment of molasses with H_2SO_4 and $K_4Fe(CN)_6$ on ethanol production by different yeast strains was studied in order to find an effective method to reduce the load of various inhibitory substances and to select a suitable yeast strain for fermentation of pre-treated molasses. Pre-treatment resulted in decreased level of inhibitory substances like Ca, Cu, Fe in the molasses solution with improved ethanol production. Strain 20 was best among the tested strains with all pre-treatments. The inhibitory effect of these constituents was confirmed by supplementation of synthetic medium with residues from different pre-treatments and inhibitory level for various constituents was found to be Ca > 0.5%, iron > 4.6 ppm and Cu > 5.4 ppm (Yadav *et al*., 1997).

Rekha Dabas *et al*. (1997) used hydrolysed wheat starch as substrate for production of ethanol by using 2 strains of *Saccharomyces cerevisiae*. Wheat flour slurry (25% w/v) was gelatinized by cooking with continuous stirring followed by treatment with cellulase and protease at a concentration of 2 mg g^{-1} starch 4 h at 40^0c. Maximum saccharification of gelatinized slurry was obtained with a combination of α-amylase and amyloglucosidase at a concentration of 3.5 mg g^{-1} starch and 12% reducing sugars were obtained in 12 hours at 40^oC at pH 5. *Saccharomyces cerevisiae* strain TBY and 21 produced 6.4% and 6% (v/v) ethanol respectively in 36 h at 30^oC.

Five thermototerant strains belonging to the genera *Kluyveromyces maraxianus* (3) and *Kluyveromyces thermotolerans* (2) were studied by growing them on agar slants and in shake flasks at different temperatures using glucose medium. The yeasts were grown in the presence of the liquid of steam pre-treated soft wood as well as test the tolerance of yeast against inhibitors formed during pre-treatment of wood. The investigated yeast proved to be sensitive to inhibitors at high temperatures. *K. marxianus* Y 01070 was the most thermotolerant on glucose medium, but it was too sensitive to inhibitors (Bollo and Reczey, 2000).

A mixed culture of an amylolytic yeast strain *Sacharomyces diastaticus* and *Zymononas mobilis* was developed for improved ethanol production from liquefied cassava starch. A batch fermentation of *S. diastaticus* produced 21.1 g ethanol L^{-1} as compared to 36.5 g ethanol L^{-1} in mixed culture system with *Z. mobilis*. The ethanol yield in mixed culture of *S. diastaticus* and *Z. mobilis* (0.34 g g^{-1}) was higher than that of monoculture (0.24 g g^{-1}) of yeast. Further increase in ethanol concentration was obtained when the mixed culture system was supplemented with minerals and salts. The addition of ammonium sulphate (1 g L^{-1}) and yeast extract 10 g L^{-1}) resulted in a distinct increase in final ethanol concentration to 44.2 g L^{-1} and 54.9 g L^{-1}, respectively in a mixed culture of *Z. mobilis* and *S. diastaticus* (Amutha and Gunasekharan, 2000).

Paneer *et al.* (2001) conducted experiments for efficient ethanol production using *Zymomonas mobilis* from molasses medium. The fermentation parameters for ethanol production (sugar conc., pH, temperature and inoculum level) were optimized. Four strains of *Z. mobilis* were screened for ethanol production potential at optimized conditions (14^{o} Brix, pH 6.0, 10% v/v inoculum level and $30^{o}C$ temperature). Further studies were also carried out to test the effect of ethanol and temperature on ethanol production efficiency. Out of four tested strains, *Z. mobilis* MTCC-90 was found to be superior to tolerance to ethanol and temperature, though increase in both ethanol and temperature had adverse effect on the ethanol productivity of this microbe.

An adapted strain of yeast, *Saccharomyces cerevisiae* MKl was optimized for ethanol production from molasses. An inoculum size of 10^8 cells mL^{-1} and fermentation temperature of 30^oC were found to produce ethanol, 89.8 g L^{-1} medium with fermentation efficiency of 88%. Supplementation of soybean meal as nitrogen source improved fermentation efficiency and decreased fermentation time resulting in overall improvement in fermentation efficiency by 6.5% in case of *Saccharomyces cerevisiae* MKl over its parent (Mandeep Kaur and Kocher, 2002).

Production of ethanol from molasses using *Saccharomyces cerevisiae* and *Zymomonas mobilis* was compared. Addition of different concentrations of ethanol in the medium markedly decreased the growth of both cultures, however yeast (*Saccharomyces cerevisiae*) was found to be more tolerant to ethanol. There was no significant difference in ethanol production from molasses diluted to 5-15% (v/v), by both the cultures; however, *Saccharomyces cerevisiae* produced more ethanol at sugar concentration above 15% (v/v). The temperature of 30^oC was found to be optimum for maximum ethanol production from 20% (w/v) glucose and sucrose and 20% (v/v) molasses in 72 h (Renu Bansal and Singh, 2003).

Direct and efficient production of ethanol by fermentation from raw (corn) starch was achieved by using the yeast *Saccharomyces cerevisiae* codisplaying *Rhizopus oryzae* glucoamylase and *Streptococcus bovis* alpha amylase by using the C-terminal-half region of alpha-agglutinin and the flocculation functional domain of F10 lp as the respective anchor proteins. In 72 h, fermentation, this strain produced 61.8 g of ethanol $litre^{-1}$, with 86.5%, theoretical yield from corn starch (Shigechi *et al*., 2004).

Jerusalem artichoke mashed tubers were fermented using single yeasts and a bacterium as well as mixed culture of microorganisms. *Kulveromyces fragalis*, a yeast with an active inulinase, was used together with either a commercial distillery yeast, *Saccharomyces cerevisiae*, or the bacterium *Zymomonas mobilis*. After batch fermentation best ethanol concentration of 0.48 g g^{-1} for the mixed population and 0.46 g g^{-1} for the single population can be obtained. The theoretical yield of the mixed culture was 2 – 12% higher than for the single microorganism (Szambelan *et al*., 2004).

CHAPTER – III

MATERIALS AND METHODS

The material and the methods adopted during the course of investigation are presented.

3.1 LOCATION OF THE EXPERIMENT

Experiment was conducted in the Department of Agricultural Microbiology & Bioenergy, Department of Horticulture, College of Agriculture and Post Graduate & Research Centre, ANGRAU, Rajendranagar, Hyderabad.

3.2 SUBSTRATES USED FOR FERMENTATION

Over ripened mangoes and tomatoes were used and they were procured from local market.

3.3 CHEMICALS

All chemicals used in the experimentation and analysis were of A.R. grade, purchased from standard chemical companies. The dehydrated media and chemicals of Himedia and Rolex were used for growing and maintenance of yeast cultures.

3.4 ISOLATION OF YEAST

The yeast cultures were isolated from soil obtained from different fruit and vegetable processing industries, dump yards and also from molasses, toddy and spoiled coconut water following streak plate method.

The samples were inoculated on to Sabroud dextrose agar medium and incubated at 37^{o}C for 24-48 hrs (Subhash Reddy, 2004).

The cultures isolated from different sources were named as:

Spoiled coconut water	AM-111
Tomato processing waste	AM-112
Molasses	AM-113
Mango processing waste	AM-114
Toddy	AM-115
Guava processing waste	AM-116
Starch Processing Waste	AM-117

3.5 SCREENING OF THE ISOLATES FOR AMYLOLYTIC AND PECTINOLYTIC ACTIVITIES

The isolates obtained from different samples were checked for both amylolytic and pectinolytic activities by using specific media.

For amylolytic activity the medium used was:

Starch	20 g
Yeast extract	10 g
Peptone	10 g
Agar	20 g
Distilled water	1 L
pH =	6.0 $\pm$ 0.2

and the cultures were screened by using Iodine as a reagent.

For pectinolytic activity MPS medium (dehydrated media) of Himedia chemicals was used and the cultures were screened using 1% aqueous hexadecyl trimethyl ammonium bromide (Cetrimide).

Composition of MP-5 Medium

Ingredients	g/L
Pectin	5.00
MonoPotassium Disodium Hydrogen Phospate	6.00
Ammonium Sulphate	2.00
Yeast Extract	1.00
Magnesium Sulphate	0.20
	mg/L
Ferrous Sulphate	1.00
Calcium Chloride	1.00
Boric Acid	0.001
Manganese Sulphate	0.001
Zinc Sulphate	0.007
Copper Sulphate	0.005
Molybdenum Trioxide	0.001
Agar	15.0 g/L
Final pH (at 25 ^{0}c)	5.0±0.2

3.6 SCREENING OF ISOLATES AND STANDARD CULTURES FOR ALCOHOL PRODUCTION

All the isolates including standard cultures obtained from NCIM, IMTECH were screened for alcohol production by taking sugar solution (25^{o} Brix) as the substrate. 200 ml of sugar solution was used for fermentative production of alcohol. All the bottles were pasteurized at 82°C for 30 minutes and rapidly cooled to room temperature. The yeast isolates were transferred to the fermentation bottles containing 200 ml of sugar solution, 2 g of yeast extract powder, 100 ppm of KMS and yeast inoculum was added at the rate of 5% under aseptic conditions.

3.7 STANDARD CULTURES

Two standard ethanol producing yeast strains viz., *Saccharomyces cerevisiae* MTCC-172 and *Saccharomyces cerevisiae* NCIM-3095 were obtained from (IMTECH) Institute of Microbial Technology, Chandigarh and (NCL) National Chemical Laboratories, Pune respectively. Fermentative production of ethanol from over ripened mango and tomato was tested using one of the two standard cultures and one of the local yeast isolated AM-113 from this laboratory.

The cultures were maintained in growth medium No.5 for MTCC-172 and MPYG medium for NCIM-3095 as per the instructions given by the IMTECH and NCL.

Composition of growth medium No.5

Dextrose	– 20 g
Yeast extract	– 20 g
Peptone	- 10 g
Agar	- 20 g
Distilled water	- 1 L
pH	- 6.0 $\pm$ 0.2

Composition of MPYG medium

Maltose	- 3.0 g
Yeast extract	- 20 g
Peptone	- 5 g
Glucose	- 10 g
Agar	- 20 g
Distilled water	- 1 L
pH	- 6.4 – 6.8

The yeast cultures were grown at 30^{o}C by incubating for 48 hours under aerobic conditions. Stock cultures of yeasts were maintained in agar slant method by sub culturing at 6-8 week interval.

3.8 EXPERIMENTAL DETAILS

Experiment	**-**	**1**
Total treatments	-	14
Substrates	-	Mango
Yeast cultures	-	*Saccharomyces cerevisiae* MTCC-172 (yeast culture 1 Y1) Local yeast isolate AM-113 (yeast culture2-Y2)
Replications	-	2

Treatments

T1	Substrate + Pectinase + yeast culture 1
T2	Substrate + Amylase + yeast culture 1
T3	Substrate + Pectinase + Amylase + yeast culture 1
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1
T5	Substrate + Without pectinase and amylase + yeast culture 1

T6	Substrate + Sugar + years culture 1
T7	Only sugar + yeast culture 1
T8	Substrate + Pectinase + yeast culture 2
T9	Substrate + Amylase + yeast culture 2
T10	Substrate + Pectinase + Amylase + yeast culture 2
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2
T12	Substrate + without pectinase and amylase + yeast culture 2
T13	Substrate + Sugar + yeast culture 2
T14	Only sugar + yeast culture 2

Experiment - 2

Total treatments	-	14
Substrates	-	Tomato
Yeast cultures	-	Saccharomyces cerevisiae-MTCC-172 (yeast culture 1 Y1) Local yeast isolate AM-113 (yeast culture 1 - Y2)
Replications	-	2

Treatments

T1	Substrate + Pectinase + yeast culture 1
T2	Substrate + Amylase + yeast culture 1
T3	Substrate + Pectinase + Amylase + yeast culture 1
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1
T5	Substrate + Without pectinase and amylase + yeast culture 1
T6	Substrate + Sugar + years culture 1
T7	Only sugar + yeast culture 1
T8	Substrate + Pectinase + yeast culture 2
T9	Substrate + Amylase + yeast culture 2
T10	Substrate + Pectinase + Amylase + yeast culture 2
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2
T12	Substrate + Without pectinase and amylase + yeast culture 2
T13	Substrate + Sugar + yeast culture 2
T14	Only sugar + yeast culture 2

3.9 FERMENTATIVE PRODUCTION OF ALCOHOL FROM MANGO AND TOMATO

3.9.1 Preparation of over ripened mango for fermentation

The over ripened mangoes were collected from the local market. They were washed with potable water and then used for preparation of pulp. The pulp was homogenized in a mixer and then diluted by using water to reduce the viscosity of the pulp and the brix was adjusted to 15^0 in all the treatments except the treatments $T_{4,}$ $T_{6,}$ T_{11} and T_{13} where the brix was 20^0 by addition of sugar solution (1:1 water and sugar) and used as substrate in different treatments as mentioned in experimental details.

3.9.2 Preparation of over ripened tomato for fermentation

The over ripened tomatoes collected from local market were washed properly and they were homogenized in a mixer. The homogenous material was used as substrate in treatments as mentioned in experimental details.

3.9.3 Preparation of bottles for fermentation

250 ml of corning glass bottles were rinsed with chromic acid followed by detergent wash. They were washed with tap water thoroughly followed by rinsing with distilled water. The bottles were sterilized in the oven (dry sterilization method) at 160^oC for 60 minutes.

3.9.4 Preparation of inoculum

Freeze dried culture in lyophilized vials of MTCC-172 were reconstituted by inoculating into the specified liquid broth and incubated at 30^oC for 48 hrs. After sufficient growth a loopful was streaked onto agar medium. After verifying the purity of culture by streak plate method, they were inoculated into ten ml of G.M.No.5 broth and incubated at 30^{o}C for 48 hours. After 48 hrs of incubation, 10 ml of the culture was transferred to 100 ml of sterilized growth medium No.5 broth and incubated at 30^{o}C for 48 hours to get turbid culture.

AM-113 i.e., local isolate which was purified earlier was inoculated into 10 ml sterile sabroud dextrose broth and incubate at 30^{o}C for 48 hrs. After 48 hours of incubation a loopful of this SD both was transferred to 100 ml of sterilized SD broth and incubated at 30^{o}C for 48 hrs to get turbid culture. This turbid cultures containing nearly 1.0 x 10^{7} cells/ml were used for inoculation into the must for fermentative production of ethanol.

3.9.5 Preparation of must

The must was first homogenized uniformly in a mixer and the brix was adjusted to 15^{o} in all the treatments and specifically enzymes viz., Amylase and pectinase were added individually and in combination in required treatments. Sugar solution which was prepared with sterile distilled water in the ratio of 1:1 was also added in required treatments and the brix was raised to 20^{o}.

3.9.6 Optimization of enzyme concentration for treating pulp

The enzyme concentrations were estimated by taking 0.5, 1.0, 1.5 and 2.0% of amylase and pectinase individually and in combination in both tomato and mango juices.

Amylase preparation containing different concentrations (0.5, 1, 1.5 and 2%) was used with substrates over ripened tomato and mango pulp. The preliminary experiments were conducted to know the optimum concentration of amylase and for treatment time suitable for increasing the sugar content. Accordingly, pre-treatment of substrates was determined to be with 2% amylase for 12 hours.

Pectinase preparation containing different concentrations (0.5, 1.0, 1.5 and 2%) was used with substrates, over ripened tomato and mango pulp. The preliminary experiments were conducted to know the optimum percent of pectinase suitable for increasing the flowability and accordingly pre-treatment of substrates was done with 2 % pectinase for 12 hours.

3.9.7 Pasteurization of the musts

pH of the must was adjusted to 5.0. Musts were pasteurized at 82^{o}C for 30 minutes in water bath. The pasteurized bottles with musts were cooled to room temperature used for fermentative production of alcohol.

3.9.8 Inoculation of the musts

Yeast cultures were added to the fermentation bottles containing 200 ml of the musts (containing either over ripened mango or tomato) at the rate of 5% inoculum under aseptic conditions.

3.9.9 Fermentation of the musts

The inoculated musts (mango and tomato) in the fermentation bottles were kept in orbital shaker at room temperature (28-30^{o}C) for 3 days. Samples were collected every 24 hours and analyzed for ethanol, total and reducing sugars content.

3.10 METHODS OF ANALYSES

3.10.1 Estimation of alcohol

Alcohol content was estimated by Colorimetry as per the procedure of Gopal Reddy *et a*l., (2005).

3.10.2 Estimation of total and reducing sugars

Total and reducing sugars were estimated by Nelson and Somayogi method (Ranganna 1979).

3.10.3 Screening of yeast isolates for amylolytic and pectinolytic activities

For testing amylolytic activity of yeast strains medium with starch was used.

The composition of the medium was starch – 20 g, peptone – 10 g, yeast extract – 10 g, agar – 20 g, distilled water – 1L and pH adjusted to 6.0 $\pm$ 0.2. The isolates were streaked on the agar plates by streak plate method and incubated at 30^{o}C for 48 – 72 hours. Amylolytic activity was tested by using Iodine solution, which reacts with starch to give a deep blue color.

When isolates utilize starch the area around the colonies will be colorless as the starch is degraded by the culture and starch is not available to the iodine for reaction.

For pectinolytic activity MP-5 medium was used. The isolates were streaked on agar plates by streak plate method and incubated at 30^{o}C for 3-4 days. The isolates were then screened for pectinolytic activity by adding 1% aqueous hexadecyl trimethyl ammonium bromide (cetrimide) on to the plate.

Cetrimide reacts with pectins and forms precipitates when culture utilize the pectins in the medium, the area around the colonies will not show precipitation, while unutilized pectin reacts with cetrimide and shows precipitation.

3.11 STATISTICAL ANALYSIS

The data were subjected to statistical analysis as per the procedure outlined by Panse and Sukhatme (1967). The design adopted was completely randomized design.

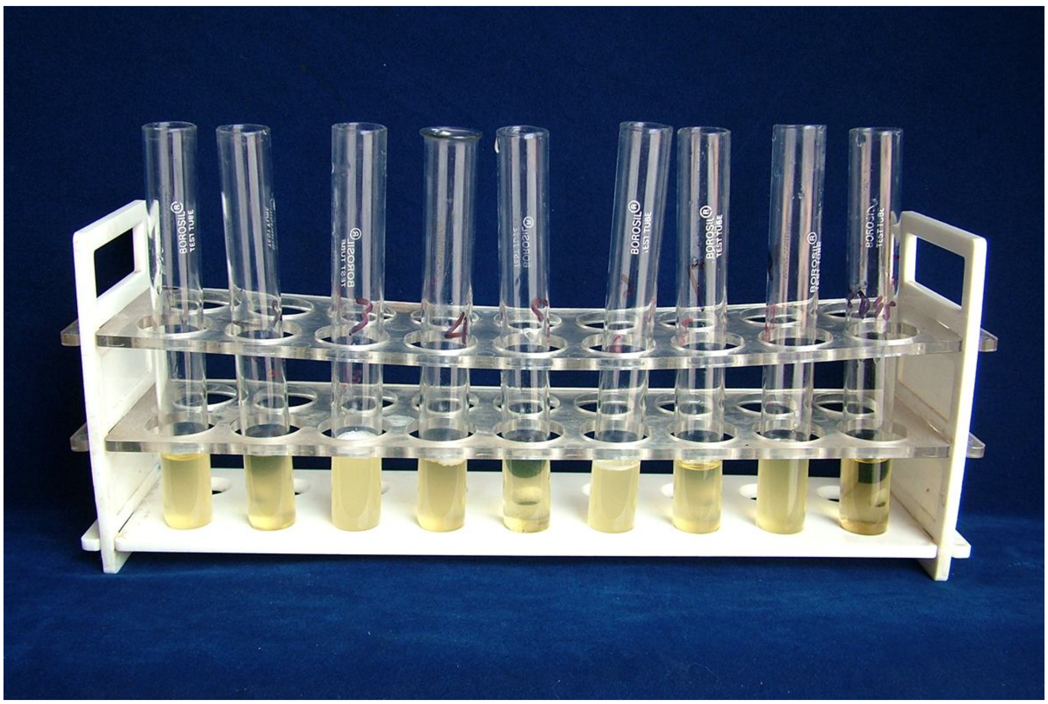


Plate: 1 Screening of Yeast Cultures for Alcohol Production

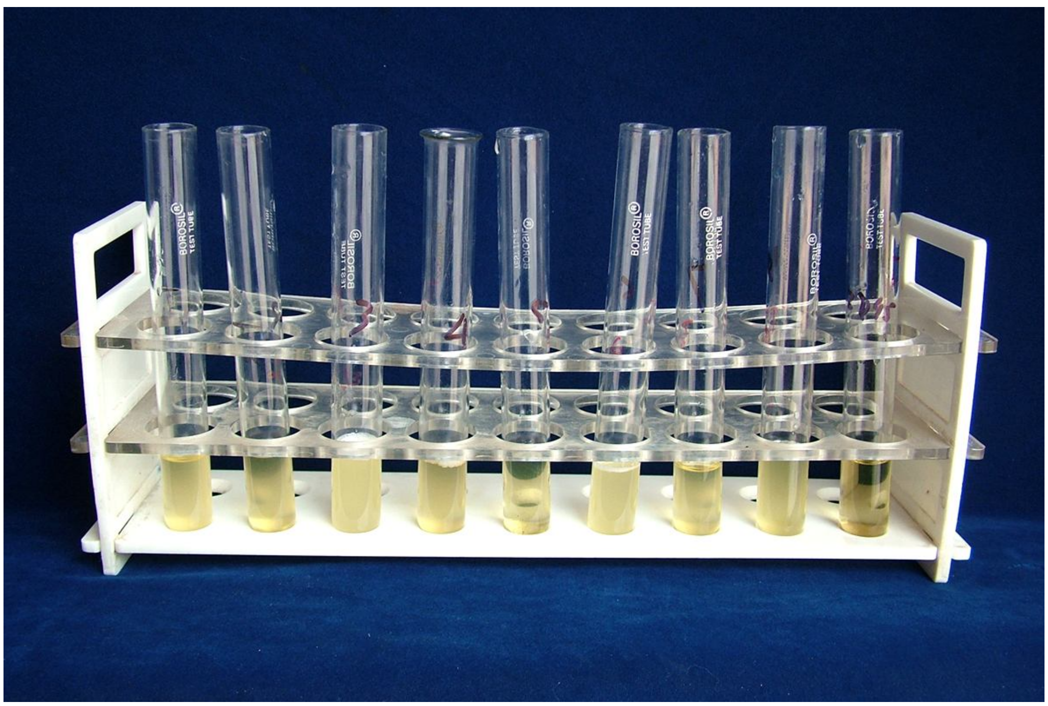
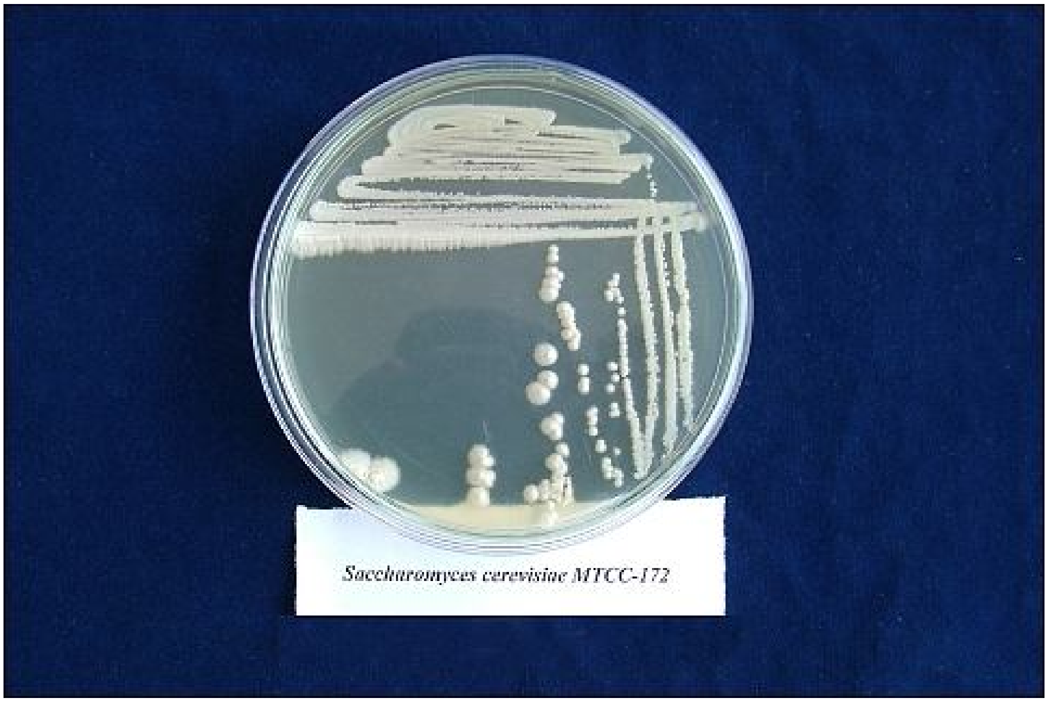


Plate: 1 Screening of Yeast Cultures for Alcohol Production



Colony Morphology of *Saccharomyces Cerevisiae* MTCC-172

Plate: 4 Colony Morphology of *Saccharomyces Cerevisiae* NCIM-3095

Plate: 3 Fermentative Production of Alcohol from over ripened Tomato Pulp

Plate: 2 Fermentative Production of Alcohol from over ripened Mango Pulp

Plate: 3 Fermentative Production of Alcohol from over ripened Tomato Pulp

CHAPTER – IV

RESULTS

Yeasts were isolated from different sources viz., spoiled coconut water, mango processing waste, guava processing waste, molasses, tomato processing waste etc. They were numbered as

AM-111	-	Spoiled coconut water
AM-112	-	Tomato processing waste
AM-113	-	Molasses
AM-114	-	Mango processing waste
AM-115	-	Toddy
AM-116	-	Guava processing waste
AM-117	-	Starch Processing Waste

4.1 CULTURAL CHARACTERS

The cultural characteristics of the yeast isolates and the standard cultures of *S. cerevisiae* MTCC-172 and *S. cerevisiae* NCIM-3095 were presented in the Table 1.

4.2 CELL MORPHOLOGY

The cell morphology of the yeast isolates and the standard cultures was observed using a compound microscope. Majority of the cultures showed round shaped cells except two isolates AM-112 and AM-114, which exhibited elliptical shape. The cell size for different cultures varied from 3.08 to 5.87 μm (Table 2).

4.3 PECTINOLYTIC ACTIVITY

Yeast isolates were screened for pectinolytic activity using MP-5 medium. Except the isolates AM-113, AM-115 and AM-117 which appeared to show slight pectinolytic activity while others did not have pectinolytic activity (Table 3).

4.4 AMYLOLYTIC ACTIVITY

Amylolytic activity of the isolates were examined growing on amylolytic medium with starch as substrate and it was observed that none of the yeast isolates had ability to degrade starch which is present in the medium (Table 4).

4.5 SCREENING OF YEAST ISOLATES AND STANDARD CULTURES FOR UTILIZATION OF SUGARS

All the isolates and standard cultures were screened for utilization of the sugars. The data in table 5 indicate that standard culture MTCC-172 utilized all sugars except lactose whereas yeast isolate AM-113 utilized all sugars except lactose, xylose and melibiose.

4.6 SCREENING OF YEAST ISOLATES AND STANDARD CULTURES FOR ALCOHOL PRODUCTION

All the isolates and the standard cultures were tested for the ability to produce alcohol fermentatively using sugar as substrate.

The data in Table 6 indicate that the standard culture MTCC-172 showed high ethanol (80.00) producing ability followed by AM-113, NCIM-3095 and AM-112. The yeast isolate AM-111 formed lowest alcohol (54.00). However both the standard cultures MTCC-172 and NCIM 3095 produced more alcohol compared to the isolates AM-111 to AM-117.

4.7 EFFECT OF ENZYME TREATMENT ON MANGO PULP

The mango pulp was treated with the enzymes viz., pectinase and amylase in single and in combination of both the enzymes to improve the flowability of the fermentation and the results after treating the pulp with the enzymes for 12 h are mentioned in the Table 7. The flowability after treating with pectinase and in combination of both pectinase and amylase were increased in the pulp. There is no change in the pulp without the treatments and it was viscous.

4.8 EFFECT OF ENZYME TREATMENT ON PULP OF TOMATO

Similarly the tomato pulp was also treated with the different combinations of enzymes viz., pectinase, amylase, pectinase and amylase. The effect of different treatments are shown in Table 8.

The flowability of the pulp after treating with the enzymes were increased in all the treatments. However, flowability was at maximum where amylase and pectinase were used.

4.9 CHANGE IN ^{O}BRIX OF PULP OF MANGO ON ENZYME TREATMENT

Since the combined treatment of pulp with pectinase and amylase was found to be better in terms of flowability of pulp, the pulp of mango was treated with the different concentration of enzymes, amylase and pectinase in the range of 0.5 to 2.0%. There was a increase in brix from 14.5^{o} to 18.5^{o} (Table 9). Maximum increase in brix of pulp was found with 2% concentration of pectinase and amylase.

4.10 CHANGE IN ^{O}BRIX OF PULP OF TOMATO ON ENZYME TREATMENT

The pulp of tomato was also treated with enzymes amylase and pectinase in the range of 0.5 to 2.0%. There was increase in brix from 4.5^{o} to 9^{o} (Table 10). The brix values of the pulp was higher with 2% of pectinase and amylase.

4.11 ALCOHOL CONTENT IN FERMENTED MANGO PULP

4.11.1 Alcohol content on 24 h of fermentation

The data pertaining to fermentative production of alcohol from over ripened mango pulp after 24 h of inoculation of yeast cultures are presented in Table 11.

There was a significant difference in alcohol content of mango due to different treatments. The maximum alcohol content (74.75) was recorded with T_7 (Sugar + Y_1), which was significantly superior to the rest of the treatments but on par with T_{14} (Sugar + Y_2). However alcohol content was lowest in T_{12} (20.22) (Substrate + Y_2) but on par with T_5 (Substrate + Y_1).

Though alcohol content in T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1) was lower than in T_7 and T_{14} but it was higher than in treatments T_3 (Substrate + Pectinase + Amylase + Y_1), T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2), T_2 (Substrate + Amylase + Y_1) and T_{10} (Substrate + Pectinase + Amylase + Y_2), which were on par.

When yeast cultures *S. cerevisiae* MTCC-172 and the yeast isolate AM-113 were compared in all the treatments, *S. cerevisiae* MTCC-172 was found to be better than the local yeast isolate.

4.11.2 Alcohol content on 48 h of fermentation

Fermentation was continued for 48 h and the samples were collected and analyzed for alcohol content. The data were presented in the Table 12.

There was a significant difference in alcohol content between the treatments. The maximum alcohol content (73.00) was noticed with T_7 (Sugar + Y_1) which was significantly superior to other treatments but on par with T_{14} (Sugar + Y_2).

The minimum alcohol content (19.52) was noticed with T_{12} (Substrate + Y_1) but on par with T_5 (Substrate + Y_1) and T_8 (Substrate + Pectinase + Y_2).

However alcohol content in T_3 (Substrate + Pectinase + Amylase + Y_1) was lower than in T_7 and T_{14} but it was higher than T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1), T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2), T_3 (Substrate + Pectinase + Amylase + Y_1), T_2 (Substrate + Amylase + Y_1) and T_{10} (Substrate + Pectinase + Amylase + Y_2), which were on par. But there was decrease in alcohol content from 24 h to 48 h.

4.11.3 Alcohol content on 72 h of fermentation

Alcohol content was estimated from the fermented medium after 72 h of inoculation of yeast cultures and data presented in Table 13.

There was a significant difference in alcohol content of mango on 72 h of fermentation in between the treatments. The highest amount of alcohol (70.14) was recorded with T_7 (Sugar + Y_1) which was significantly superior to other treatments but on par with T_{14} (Sugar + Y_2). However, lowest alcohol content (18.50) was noticed with T_{12} (Substrate + Y_2) but on par with T_5 (Substrate + Y_1) and T_8 (Substrate + Pectinase + Y_2).

When the data on alcohol content on three days are compared, alcohol content decreased from 1st to 3rd day with both yeast cultures MTCC-172 and AM 113. Though the alcohol production was maximum in 24 h in almost all treatments, on all days the highest alcohol was found in treatments, where pectinase and amylase and sugars are used while lowest alcohol content was found in treatments where only mango pulp is used without supplementation of enzymes and sugar.

4.12 REDUCING SUGARS IN MANGO PULP AFTER FERMENTATION

4.12.1 Reducing sugars on 24 h of fermentation

The data pertaining to reducing sugars on 24 h of fermentation of mango pulp is presented in Table 14.

There was a significant difference in reducing sugars content due to different treatments. The minimum reducing sugars content (1.41) was observed with T_9 (Substrate + Amylase + Y_2) which were on par with T_5 (Substrate + Y_1), T_{13} (Substrate + Y_2) and T_8 (Substrate + Pectinase + Y_2).

The maximum reducing sugars (3.81) content was noticed with T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1), which was significantly superior to the rest of the treatments.

However reducing sugar content in T_7 (Sugar + Y_1) was lower than in treatment T4 but was higher than in treatments T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2) and T_{14} (Sugar + Y_2).

4.12.2 Reducing sugars on 48 h of fermentation

The data from Table 15 revealed that, there was a significant difference in reducing sugars of mango on 48 h of fermentation due to different treatments.

The lowest reducing sugars content (0.27) was observed with T_{12} (Substrate + Y_2) which were on par with T_{13} (Substrate + Sugar + Y_2) and T_5 (Substrate + Y_2).

The highest reducing sugars content (2.49) was recorded with T_{11} (Substrate + Pectinase + Amylase + Y_2), which was significantly superior to rest of the treatments but on par with T_{14} (Sugar + Y_2) and T_7 (Sugar + Y_1).

The reducing sugars content decreased from 24 h - 48 h for both the yeast cultures MTCC-172 and AM-113.

4.12.3 Reducing sugars on 72 h of fermentation

The data in Table 16 indicate that there was a significant difference among the treatments in terms of reducing sugars content.

The reducing sugars content was reduced to zero in T_5 (Substrate + Y_1), T_6 (Substrate + Sugar + Y_1), T_{12} (Substrate + Y_1) and T_{13} (Substrate + Sugar + Y_2).

The maximum reducing sugars content (1.97) was found with T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1) which was significantly superior to other treatments but on par with T_{14} (Sugar + Y_2), T_7 (Sugar + Y_1) and T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2).

The content of reducing sugars were decreased gradually from 24 h to 72 h during the process of fermentation. In treatments T_5, T_6, T_{12} and T_{13}, the reducing sugars were utilized completely by 72 h.

4.13 TOTAL SUGARS IN MANGO PULP AFTER FERMENTATION

4.13.1 Total sugars in mango pulp after 24 h of fermentation

There was a significant difference in total sugars content of mango due to treatments. The data presented in Table 18.

The lowest total sugars content (2.43) was found with T_{12} (Substrate + Y_2), which were on par with T_5 (Substrate + Y_1), T_{13} (Substrate + Sugar + Y_2), T_6 (Substrate + Sugar + Y_1), T_2 (Substrate + Amylase + Y_1), T_9 (Substrate + Amylase + Y_2) and T_8 (Substrate + Pectinase + Y_2). The maximum total sugars (4.75) content was recorded with T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1), which was significantly superior to rest of the treatments, which were on par with T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2) and T_{14} (Sugar + Y_2).

Though total sugars content in T_3 (Substrate + Pectinase + Amylase + Y_1) was lower than the treatments T_4, T_{11} and T_{14} but was higher than in treatments T_{10} (Substrate + Pectinase + Amylase + Y_2) and T_1 (Substrate + Pectinase + Y_1).

4.13.2 Total sugars in mango pulp after 48 h of fermentation

The data pertaining to total sugars after 48 h of fermentation were mentioned in the Table 18.

There was a significant difference due to treatments. The minimum total sugars content (0.75) was recorded with T_{12} (Substrate + Y_2) which were on par with T_5 (Substrate + Y_1), T_{13} (Substrate + Sugar + Y_2) and T_6 (Substrate + Sugar + Y_2). The maximum total sugars content (3.63) was found in the treatments T_4 (Substrate + Pectinase + Amylase + Y_1) which was superior to rest of the treatments but on par with T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2) and T_{14} (Sugar + Y_2).

Though total sugars in T_{10} (Substrate + Pectinase + Amylase + Y2) was lower than in the treatments T_4, T_{11} and T_{14} but it was higher than in the treatment T_3 (Substrate + Pectinase + Amylase + Y_1) which were on par.

4.13.3 Total sugars in mango pulp after 72 h of fermentation

Total sugars content was analyzed on 72 h of fermentation and the results were presented in the Table 19. There was a significant difference among the treatments.

Total sugars content was reduced to zero level in T_5 (Substrate + Y_1), T_6 (Substrate + Sugar + Y_1), T_{12} (Substrate + Y_2) and T_{13} (Substrate + Sugar + Y_2) where the total sugars were completely utilized by the microbes. However high amount of total sugars (2.50) was noticed with T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1) and (Sugar + Y_1) which were significantly superior to rest of the treatments but on par with T_{14} (Sugar + Y_2) and T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2)

The total sugars content were reduced gradually from 24 h to 72 h for both the yeast cultures MTCC-172 and AM-113.

4.14 ALCOHOL CONTENT IN FERMENTED TOMATO PULP

4.14.1 Alcohol content on 24 h of fermentation

Alcohol content of tomato pulp after 24 h inoculation of yeast cultures were mentioned in the Table 20.

There was a significant difference between the treatments in alcohol content of tomato pulp.

The highest alcohol content (81.00) was noticed with T_7 (Sugar + Y_1), which was superior to rest of the treatments but on par with T_{14} (Sugar + Y_2).

The lowest alcohol content (15.00) was recorded with T_{12} (Substrate + Y_2) but on par with T_5 (Substrate + Y_1), T_1 (Substrate + Pectinase + Y_1) and T_8 (Substrate + Pectinase + Y_2).

However alcohol content in T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1) was lower than in T_7 and T_{14} but it was higher in treatments T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2), T_3 (Substrate + Pectinase + Amylase + Y_1) and T_{10} (Substrate + Pectinase + Amylase + Y_2) which were on par.

When yeast cultures *S. cerevisiae* MTCC-172 and the yeast isolate AM-113 were compared, except in treatments T_9 and T_{13}, *S. cerevisiae* MTCC-172 was found to be better.

4.14.2 Alcohol content on 48 h of fermentation

Fermentation was continued for 2^{nd} day and the samples were analyzed for alcohol content. The data were depicted in the Table 21.

There was a significant difference in alcohol content due to treatments. The maximum alcohol content (78.00) was recorded with T_7 (Sugar + Y_1) which was significantly superior to rest of the treatments but on par with T_{14} (Sugar + Y_2). The lowest alcohol content (18.25) was seen in T_{12} (Substrate + Y_2) but on par with T_1 (Substrate + Pectinase + Y_1), T_5 (Substrate + Y_1) and T_8 (Substrate + Pectinase + Y_2).

However alcohol content in T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1) was lower than the treatments T_7 and T_{14} but higher than in T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2), T_3 (Substrate + Pectinase + Amylase + Y_1) and T_{10} (Substrate + Pectinase + Amylase + Y_2) which were on par.

The alcohol content increased from 24 h to 48 h in all the treatments except in T_7 and T_{14} where decrease in alcohol content was observed.

4.14.3 Alcohol content after 72 h of fermentation

The data pertaining to alcohol content after 72 h of inoculation of yeast cultures was presented in Table 22.

There was a significant difference in alcohol content of tomato on 3rd day of fermentation due to the treatments. The maximum alcohol content (75.00) was recorded with T_7 (Sugar + Y_1) which was significantly superior to the rest of the treatments but on par with T_{14} (Sugar + Y_2) and T_3 (Substrate + Pectinase + Amylase + Y_1). However minimum alcohol content (14.75) was noticed with T_{12} (Substrate + Y_2) but on par with T_5 (Substrate + Y_1), T_1 (Substrate + Pectinase + Y_1) and T_8 (Substrate + Pectinase + Y_2).

Though the alcohol content in T_3 (Substrate + Pectinase + Amylase + Y_1) was lower than in T_7 and T_{14} but was more than the T_{10} (Substrate + Pectinase + Amylase + Y_2), T_{11} (Substrate + Pectinase + Amylase + Sugar + Y_2) and T_4 (Substrate + Pectinase + Amylase + Sugar + Y_1).

The alcohol content first increased from 24 h to 48 h and then decreased during 72 h except in treatments T_3 and T_{11} with both the cultures.

4.15 TOTAL AND REDUCING SUGARS IN TOMATO PULP AFTER FERMENTATION

Total and reducing sugars were almost reduced to zero levels in all the treatments except in T_3, T_4, T_7, T_{10}, T_{11} and T_{14} wherein also the sugars were utilized completely by next 24 hours i.e., by 48 hours of fermentation.

Table: 1 Cultural Characters of Yeast Isolates and Standard Cultures

S.No	Yeast Isolates	Colony Morphology
1.	AM-111	Light dull white colonies with slight elevation. Round darkened center.
2.	AM-112	Creamish white colonies without elevation. Irregular concentric ring with darkened center.
3.	AM-113	Creamish white colonies with elevation and round concentric rings.
4.	AM_114	Creamish white colonies with elevation and round concentric rings but wrinkles are seen.
5.	AM-115	Dark brown colonies with slight elevation and irregular smooth colonies with darkened center.
6.	AM-116	Creamish yellow colonies with elevated center and irregular colonies, concentric rings and darkened center.
7.	AM-117	Brown colonies with smooth borders and irregular colonies with darkened center.
8.	MTCC-172	Creamish white colonies with elevation. Irregular colonies with irregular borders and darkened center.
9.	NCIM-3095	Creamish white colonies with elevation. Irregular colonies with irregular borders.

Table: 2 Morphology of Yeast Isolates and Standard Cultures

Yeast Isolates	Shape	Colour	Cell Size (μm)
AM-111	Round	Dull White	4.75
AM-112	Elliptical	Creamish White	5.87×2.2
AM_113	Round	Creamish White	4.4
AM-144	Round	Creamish White	3.85
AM-115	Round	Dark Brown	3.08
AM-116	Round	Creamish Yellow	4.16
AM-117	Elliptical	Dark Brown	4.4×2.2
MTCC-172	Round	Creamish White	5.28
NCIM-3095	Round	Creamish White	4.95

Table: 3 Screening of Yeast Isolate for Pectinolytic Activity

Yeast Isolates	Pectinolytic Activity
AM-111	–
AM-112	–
AM-113	±
AM-114	–
AM-115	±
AM-116	–
AM-117	±

± Slight Pectinolytic Activity
– No Pectinolytic Activity

Table 4: Screening of Yeast Isolates for Amylolytic Activity

Yeast Isolates	Amylolytic Activity
AM-111	–
AM-112	–
AM-113	–
AM-114	–
AM-115	–
AM-116	–
AM-117	–

– No amylolytic Activity

Table: 5 Utilization of Different Sugars by Yeast Isolates and Standard Cultures

Sugars	AM-111	AM-112	AM-113	AM-114	AM-115	AM-116	AM-117	MTCC-172	NCIM-3095
Lactose	—	—	—	—	—	—	—	—	—
Xylose	—	—	—	—	—	—	—	+	—
Maltose	+	—	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+
Galactose	—	—	+	—	+	+	—	+	+
Raffinose	+	—	+	—	+	+	—	+	+
Trehalose	—	—	+	—	—	+	—	+	—
Melibiose	—	—	—	—	—	—	—	+	—
Sucrose	+	—	+	+	+	+	—	+	+
L-Arabinose	—	—	+	—	—	—	—	+	+
Mannose	+	+	+	+	+	+	+	+	+

+ Utilization of Sugars
— No Utilization of Sugars

Table: 6 Screening of Yeast Isolates and Standard Cultures for Alcohol Production

Yeast Isolates	Alcohol Content g/L on Day1	Alcohol Content g/L on Day2	Mean
AM-111	54.00	53.00	53.50
AM-112	70.00	72.00	71.00
AM-113	74.00	72.00	73.00
AM-114	61.00	63.00	62.00
AM-115	69.00	67.00	68.00
AM-116	51.00	50.00	50.50
AM-117	57.00	55.00	56.00
MTCC-172	80.00	78.00	79.00
NCIM-3095	72.00	71.00	71.50
Mean	65.33	64.55	64.94

	Days	Treatments	Days X Treatments
SEm±	0.98	2.09	2.96
CD AT 5%	NS	6.22	NS

Table: 7 Effect of Enzyme Treatment on Mango Pulp

S.No.	Tretments	Viscosity
1	Pectinase	Free Flowing
2	Amylase	Slightly Viscous
3	Pectinase+Amylase	Free Flowing
4	Without Enzymes	Viscous

Table: 8 Effect of enzyme treatment on Tomato Pulp

S.No.	Tretments	Viscosity
1	Pectinase	Highly Free Flowing
2	Amylase	Free Flowing
3	Pectinase+Amylase	Highly Free Flowing
4	Without Enzymes	Slightly Viscous

Table: 9 Change in Brix of Pulp of Mango on Enzyme Treatment

S.No.	Treatments	^{0}Brix
1	Control	14.5
2	0.5%Pectinase+Amylase	15.0
3	1.0%Pectinase+Amylase	15.5
4	1.5%Pectinase+Amylase	16.0
5	2.0%Pectinase+Amylase	17.0

Table: 10 Change in Brix of Pulp of Tomato on Enzyme Treatment

S.No.	Treatments	^{0}Brix
1	Control	4.5
2	0.5%Pectinase+Amylase	6.0
3	1.0%Pectinase+Amylase	6.5
4	1.5%Pectinase+Amylase	8.5
5	2.0%Pectinase+Amylase	9.0

Table: 14 Reducing Sugars Content In Mango on 24 h of Fermentation

	Treatments	Reducing Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	2.32
T2	Substrate + Amylase + yeast culture 1	1.87
T3	Substrate + Pectinase + Amylase + yeast culture 1	2.77
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	3.81
T5	Substrate + Without pectinase and amylase + yeast culture 1	1.44
T6	Substrate + Sugar + years culture 1	1.57
T7	Only sugar + yeast culture 1	3.43
T8	Substrate + Pectinase + yeast culture 2	1.61
T9	Substrate + Amylase + yeast culture 2	1.41
T10	Substrate + Pectinase + Amylase + yeast culture 2	2.68
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	3.27
T12	Substrate + Without pectinase and amylase + yeast culture 2	1.84
T13	Substrate + Sugar + yeast culture 2	1.56
T14	Only sugar + yeast culture 2	3.25
	SEm±	0.11
	CD at 5%	0.34

Table: 15 Reducing Sugars Content In Mango on 48 h of Fermentation

	Treatments	Reducing Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	1.77
T2	Substrate + Amylase + yeast culture 1	0.68
T3	Substrate + Pectinase + Amylase + yeast culture 1	1.63
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	2.18
T5	Substrate + Without pectinase and amylase + yeast culture 1	0.33
T6	Substrate + Sugar + years culture 1	0.52
T7	Only sugar + yeast culture 1	2.30
T8	Substrate + Pectinase + yeast culture 2	0.87
T9	Substrate + Amylase + yeast culture 2	0.76
T10	Substrate + Pectinase + Amylase + yeast culture 2	1.88
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	2.49
T12	Substrate + Without pectinase and amylase + yeast culture 2	0.27
T13	Substrate + Sugar + yeast culture 2	0.41
T14	Only sugar + yeast culture 2	2.36
	SEm±	0.079
	CD at 5%	0.24

Table: 16 Reducing Sugars Content In Mango on 72 h of Fermentation

	Treatments	Reducing Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	0.23
T2	Substrate + Amylase + yeast culture 1	0.47
T3	Substrate + Pectinase + Amylase + yeast culture 1	1.31
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	1.97
T5	Substrate + Without pectinase and amylase + yeast culture 1	0.00
T6	Substrate + Sugar + years culture 1	0.00
T7	Only sugar + yeast culture 1	1.88
T8	Substrate + Pectinase + yeast culture 2	0.21
T9	Substrate + Amylase + yeast culture 2	0.45
T10	Substrate + Pectinase + Amylase + yeast culture 2	1.72
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	1.86
T12	Substrate + Without pectinase and amylase + yeast culture 2	0.00
T13	Substrate + Sugar + yeast culture 2	0.00
T14	Only sugar + yeast culture 2	1.90
	SEm±	0.06
	CD at 5%	0.19

Table: 17 Total Sugars Content In Mango on 24h of Fermentation

	Treatments	Total Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	3.15
T2	Substrate + Amylase + yeast culture 1	2.48
T3	Substrate + Pectinase + Amylase + yeast culture 1	3.80
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	4.75
T5	Substrate + Without pectinase and amylase + yeast culture 1	2.50
T6	Substrate + Sugar + years culture 1	2.75
T7	Only sugar + yeast culture 1	4.62
T8	Substrate + Pectinase + yeast culture 2	2.70
T9	Substrate + Amylase + yeast culture 2	2.47
T10	Substrate + Pectinase + Amylase + yeast culture 2	3.70
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	4.51
T12	Substrate + Without pectinase and amylase + yeast culture 2	2.43
T13	Substrate + Sugar + yeast culture 2	2.63
T14	Only sugar + yeast culture 2	4.51
	SEm±	0.15
	CD at 5%	0.46

Table: 18 Total Sugars Content In Mango on 48 h of Fermentation

	Treatments	Total Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	1.72
T2	Substrate + Amylase + yeast culture 1	1.43
T3	Substrate + Pectinase + Amylase + yeast culture 1	2.64
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	3.63
T5	Substrate + Without pectinase and amylase + yeast culture 1	0.89
T6	Substrate + Sugar + years culture 1	1.05
T7	Only sugar + yeast culture 1	3.15
T8	Substrate + Pectinase + yeast culture 2	1.62
T9	Substrate + Amylase + yeast culture 2	1.33
T10	Substrate + Pectinase + Amylase + yeast culture 2	2.98
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	3.47
T12	Substrate + Without pectinase and amylase + yeast culture 2	0.75
T13	Substrate + Sugar + yeast culture 2	0.97
T14	Only sugar + yeast culture 2	3.43
	SEm±	0.11
	CD at 5%	0.35

Table: 19 Total Sugars Content In Mango on 72 h of Fermentation

	Treatments	Total Sugars g/100g
T1	Substrate + Pectinase + yeast culture 1	0.50
T2	Substrate + Amylase + yeast culture 1	0.85
T3	Substrate + Pectinase + Amylase + yeast culture 1	2.25
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	2.50
T5	Substrate + Without pectinase and amylase + yeast culture 1	0.00
T6	Substrate + Sugar + years culture 1	0.00
T7	Only sugar + yeast culture 1	2.50
T8	Substrate + Pectinase + yeast culture 2	0.43
T9	Substrate + Amylase + yeast culture 2	0.72
T10	Substrate + Pectinase + Amylase + yeast culture 2	2.10
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	2.40
T12	Substrate + Without pectinase and amylase + yeast culture 2	0.00
T13	Substrate + Sugar + yeast culture 2	0.00
T14	Only sugar + yeast culture 2	2.48
	SEm±	0.03
	CD at 5%	0.09

Table: 11 Alcohol Content In Mango on 24h of Fermentation

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	31.60
T2	Substrate + Amylase + yeast culture 1	61.48
T3	Substrate + Pectinase + Amylase + yeast culture 1	60.67
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	64.50
T5	Substrate + Without pectinase and amylase + yeast culture 1	23.41
T6	Substrate + Sugar + years culture 1	46.71
T7	Only sugar + yeast culture 1	74.75
T8	Substrate + Pectinase + yeast culture 2	28.25
T9	Substrate + Amylase + yeast culture 2	55.52
T10	Substrate + Pectinase + Amylase + yeast culture 2	59.15
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	62.35
T12	Substrate + Without pectinase and amylase + yeast culture 2	20.21
T13	Substrate + Sugar + yeast culture 2	40.54
T14	Only sugar + yeast culture 2	69.75
	SEm±	2.32
	CD at 5%	7.05

Table: 12 Alcohol Content In Mango on 48 h of Fermentation

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	27.00
T2	Substrate + Amylase + yeast culture 1	60.00
T3	Substrate + Pectinase + Amylase + yeast culture 1	61.33
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	61.28
T5	Substrate + Without pectinase and amylase + yeast culture 1	23.00
T6	Substrate + Sugar + years culture 1	45.00
T7	Only sugar + yeast culture 1	73.00
T8	Substrate + Pectinase + yeast culture 2	25.12
T9	Substrate + Amylase + yeast culture 2	51.23
T10	Substrate + Pectinase + Amylase + yeast culture 2	57.81
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	60.50
T12	Substrate + Without pectinase and amylase + yeast culture 2	19.52
T13	Substrate + Sugar + yeast culture 2	39.00
T14	Only sugar + yeast culture 2	69.00
	SEm±	1.98
	CD at 5%	6.01

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	27.00
T2	Substrate + Amylase + yeast culture 1	57.00
T3	Substrate + Pectinase + Amylase + yeast culture 1	55.47
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	57.31
T5	Substrate + Without pectinase and amylase + yeast culture 1	21.18
T6	Substrate + Sugar + years culture 1	40.23
T7	Only sugar + yeast culture 1	70.14
T8	Substrate + Pectinase + yeast culture 2	25.12
T9	Substrate + Amylase + yeast culture 2	48.5
T10	Substrate + Pectinase + Amylase + yeast culture 2	58.00
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	60.15
T12	Substrate + Without pectinase and amylase + yeast culture 2	18.50
T13	Substrate + Sugar + yeast culture 2	38.21
T14	Only sugar + yeast culture 2	66.75
	SEm$\pm$	2.23
	CD at 5%	6.78

Table: 13 Alcohol Content In Mango on 72 h of Fermentation

Table: 20 Alcohol Content In Tomato on 24h of Fermentation

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	19.18
T2	Substrate + Amylase + yeast culture 1	27.31
T3	Substrate + Pectinase + Amylase + yeast culture 1	61.94
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	65.07
T5	Substrate + Without pectinase and amylase + yeast culture 1	16.70
T6	Substrate + Sugar + years culture 1	54.31
T7	Only sugar + yeast culture 1	81.00
T8	Substrate + Pectinase + yeast culture 2	17.00
T9	Substrate + Amylase + yeast culture 2	29.00
T10	Substrate + Pectinase + Amylase + yeast culture 2	59.12
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	64.51
T12	Substrate + Without pectinase and amylase + yeast culture 2	15.00
T13	Substrate + Sugar + yeast culture 2	55.00
T14	Only sugar + yeast culture 2	78.00
	SEm±	2.01
	CD at 5%	6.10

Table: 21 Alcohol Content In Tomato on 48 h of Fermentation

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	20.47
T2	Substrate + Amylase + yeast culture 1	31.00
T3	Substrate + Pectinase + Amylase + yeast culture 1	65.00
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	68.00
T5	Substrate + Without pectinase and amylase + yeast culture 1	19.15
T6	Substrate + Sugar + years culture 1	55.19
T7	Only sugar + yeast culture 1	78.00
T8	Substrate + Pectinase + yeast culture 2	20.23
T9	Substrate + Amylase + yeast culture 2	31.00
T10	Substrate + Pectinase + Amylase + yeast culture 2	62.15
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	67.00
T12	Substrate + Without pectinase and amylase + yeast culture 2	18.25
T13	Substrate + Sugar + yeast culture 2	54.15
T14	Only sugar + yeast culture 2	77.28
	SEm±	2.28
	CD at 5%	6.93

Table: 22 Alcohol Content In Tomato on 72 h of Fermentation

	Treatments	Alcohol Content g/L
T1	Substrate + Pectinase + yeast culture 1	20.47
T2	Substrate + Amylase + yeast culture 1	29.25
T3	Substrate + Pectinase + Amylase + yeast culture 1	69.00
T4	Substrate + Pectinase + Amylase + Sugar + yeast culture 1	67.00
T5	Substrate + Without pectinase and amylase + yeast culture 1	15.72
T6	Substrate + Sugar + years culture 1	56.44
T7	Only sugar + yeast culture 1	75.00
T8	Substrate + Pectinase + yeast culture 2	20.23
T9	Substrate + Amylase + yeast culture 2	30.00
T10	Substrate + Pectinase + Amylase + yeast culture 2	67.00
T11	Substrate + Pectinase + Amylase + Sugar + yeast culture 2	64.00
T12	Substrate + Without pectinase and amylase + yeast culture 2	14.75
T13	Substrate + Sugar + yeast culture 2	55.00
T14	Only sugar + yeast culture 2	73.00
	SEm±	2.26
	CD at 5%	6.86

CHAPTER – V

DISCUSSION

5.1 ISOLATION AND SCREENING OF YEAST ISOLATES FOR AMYLOLYTIC AND PECTINOLYTIC ACTIVITIES

The yeast cultures isolated from different sources were obtained in pure cultures following enrichment and streak plate method. The pure cultures were verified for the cell morphology, cultural characters and biochemical characters listed in Table (1, 2 & 5) Plates (3, 4 & 5).

5.2 SCREENING OF YEAST ISOLATES AND STANDARD CULTURES FOR ALCOHOL PRODUCTION

The yeast strains were screened for pectinolytic and amylolytic activity, in addition to their ability to alcohol production from sugar solution. Alcohol production was highest by MTCC-172 followed by yeast isolated from molasses. The standard yeast cultures MTCC-172, NCIM-3095 and also different yeast isolates differ significantly among themselves in alcohol production. However, the alcohol content ranged from 54 g/L to 80 g/L among the yeast cultures including the standard cultures. The data indicate clearly that the alcohol production was nearly complete in 24 h duration and the fermentation can be terminated by 24 h. Similar type of results were found from Neelima and Tauro (1983) that maximum alcohol could be produced within 24 h from sugarbeet. The yeast cultures can be rated in terms of their alcohol production in the order: MTCC-172 > AM-113 > NCIM-3095 > AM-112 > AM-115 > AM-114 > AM-117 > AM-111 > AM-116.

5.3 EFFECT OF ENZYMES, PECTINASE AND AMYLASE TREATMENTS ON PULP OF MANGO AND TOMATO

The treatment of over ripened mango and tomato with both enzymes helped in improving the brix from (14.5 to 17.0) in mango and (4.5 to 9.0) in tomato in addition to improving the flowability of the pulp by reducing the viscosity of the material. Maximum saccharification of gelatinized slurry was obtained with a combination of α-amylase and amyloglucosidase as a concentration of 3.5 mg g^{-1} starch and 12% reducing sugars were obtained in 12 hours at $40^{o}C$ at pH 5 by Rekha Dabas *et al.* (1997).

5.4 FERMENTATIVE PRODUCTION OF ALCOHOL FROM MANGO PULP

In the present study, the substrates over ripened mango pulp were used for alcohol production in the light of importance of biofuels production from the substrates which would otherwise be of no value and a cause of pollution in dumping yards. Alcohol production from such substrate [over ripened mangoes] fetch some value to the farmer in a period of glut in the market. Two yeast cultures, are obtained from IMTECH and a local yeast isolate were used to ferment the substrates to alcohol. The data presented in the tables (11, 12, 13) and figures (1& 2) indicate clearly that the standard yeast culture and isolate both showed higher alcohol production with sugar as substrate than compared to fruit pulp and also with addition of enzymes and or sugar. Relatively, the standard cultures exhibited higher alcohol production compared to local yeast isolate. Alcohol production was at maximum in 24 h in almost all treatments with both cultures. There was no increase in alcohol production further after 24 h when fermentation continued upto 48 h and 72 h.

It is clear from the data that the treatment of mango pulp with enzymes improved alcohol production from the substrate when enzymes treatment are compared to alcohol production with pectinase treatment 23 g/L, 28 g/L respectively in treatments T_1 and T_8 was on par with substrate 23 g/L, 20 g/L respectively in T_5 and T_{12} alone. However, addition of amylase alone significantly increased alcohol production to about 60 g/L, 59 g/L in T_2 and T_9 compared to 23 g/L, 20 g/L in T_1 and T_8 with substrate alone. Toyana *et al*. (1984) produced ethanol from sweet potato tubers by applying enzyme preparations. Addition of pectinase along with amylase did not improve the alcohol yields.

Addition of sugar to mango pulp increased the alcohol content significantly 46 g/L, 40 g/L in treatments T_6 and T_{13} compared to 23 g/L and 20 g/l in T_5 and T_{12} (approximately doubled). Renu Bansal and Singh (2003) obtained more ethanol production with sugar concentration above 15% v/v. However addition of sugar to enzyme treated pulp did not improve alcohol production compared to either combined addition of enzymes or amylase alone treated pulp.

Hence the data from the present study clearly indicate that the addition of pectinase or addition of sugarcane to enzyme treated pulp not really help in improving alcohol production. However, it is possible to get higher alcohol yields by supplementing the pulp with sugar or by treating the pulp with amylase alone. Therefore, exposing the pulp to amylase enzyme either by amylase enzyme addition per se or amylase production by co culturing of amylase producing yeast for fermentation of pulp to alcohol would help in production of alcohol more economically as the addition of sugar alone also improved alcohol yields but the yield improvement with amylase treatment is almost doubled alcohol production Mohamed Abouzied and Adinarayana Reddy (1986) used cocultures of *Aspergillus niger* (Amylolytic fungus) and *Saccharomyces cerevisiae* for production of ethanol from potato starch. Sharma *et al.*, (2002) isolated yeast having amylolytic activity and ethanol producing ability.

Though wine was produced from pulp of different varieties of mangoes, pulp from fruits in good condition are to be used for quality wine production. In the present study, an effort was made to produce alcohol from over ripened mangoes which cannot be used either for table purpose or for wine production. It could be much more economical if an alcohol producing yeast with amylolytic activity can be obtained and an effort was made to that effect in the investigation presented herein.

The total sugars content was followed during the fermentation period upto 72 h. It is observed that in all the treatments, with both yeast cultures, the total sugars reduced from 24 to 72 h period (Table 17, 18 and 19, Figures 3 and 4). Total sugars were reduced to about 2.5% in 24 h in the treatments, T_1, T_2, T_5 and T_6. They were higher around 4 to 4.5% in treatments T_3, T_4 and T_7. However, in treatments where only pulp or pulp supplemented with sugar, the total sugars are utilized completely by 72 h while in treatments T_3, T_4, T_7 the residual total sugars were in the range of 2 to 2.5% and in treatments T_1 and T_2 it was around 0.5%. Since, the alcohol content did not increase after 24 h upto 72 h in any of the treatments, the reduction in total sugar content can be due to the utilization of sugars for cell maintenance in both cultures without further alcohol production.

The data from Table 14, 15 and 16, Figures 5 and 6 show that the utilization of reducing sugars in fermentative production of alcohol from mango pulp. The maximum utilization was found in all treatments by 24 h though some residual reducing sugars were found upto 72 h period. The unutilized reducing sugars content varied significantly on all days among different treatments. Among the treatments with standard yeast culture, the reduction in reducing sugars is significantly lower in T_5 and T_6 compared to other treatments and the reducing sugars were completely utilized by the end of 3^{rd} day. The reduction in reducing sugars was significantly lower in treatments with pectinase, pectinase + amylase, substrate alone, substrate + sugar when yeast isolate was the culture. However, in substrate alone and substrate with sugar treatments, reducing sugars were completely utilized though there is no increase in alcohol production indicating that the sugars are utilized for cell maintenance.

The residual, reducing sugars content was the highest and on par in treatments, T_4 and T_{11} wherein sugar is supplemented to pulp with amylase + pectinase enzyme and also in sugar alone treatments T_7 and T_{14} with both cultures. However, there was no increase in alcohol content during 24 h to 72 h duration.

When residual, total sugars content was compared it was the highest and in treatments T_4 and T_{11} which were on par, wherein sugar is supplemented to pulp with amylase + pectinase enzyme and also in sugar alone treatments T_7 and T_{14} with both cultures. However, there was no increase in alcohol content during 24 h to 72 h duration.

5.5 FERMENTATIVE PRODUCTION OF ALCOHOL FROM TOMATO PULP

Tomato is one of the vegetable crops which is produced in large quantity in Andhra Pradesh and at times farmers does not even harvest the crop as the money fetched on sale of tomato harvest is far less than the amount spent only on harvesting and transport leaving aside production cost pushing the farmer into distress. Though selected tomato varieties are useful for table purposes and in preparation of processed products such as ketchups, sauces, purees etc, local varieties for several reasons are not used for further processing. Keeping in view of the facts mentioned above, the local variety of tomato is used for fermentative production of alcohol using yeast cultures MTCC-172 and a local yeast isolate AM-113.

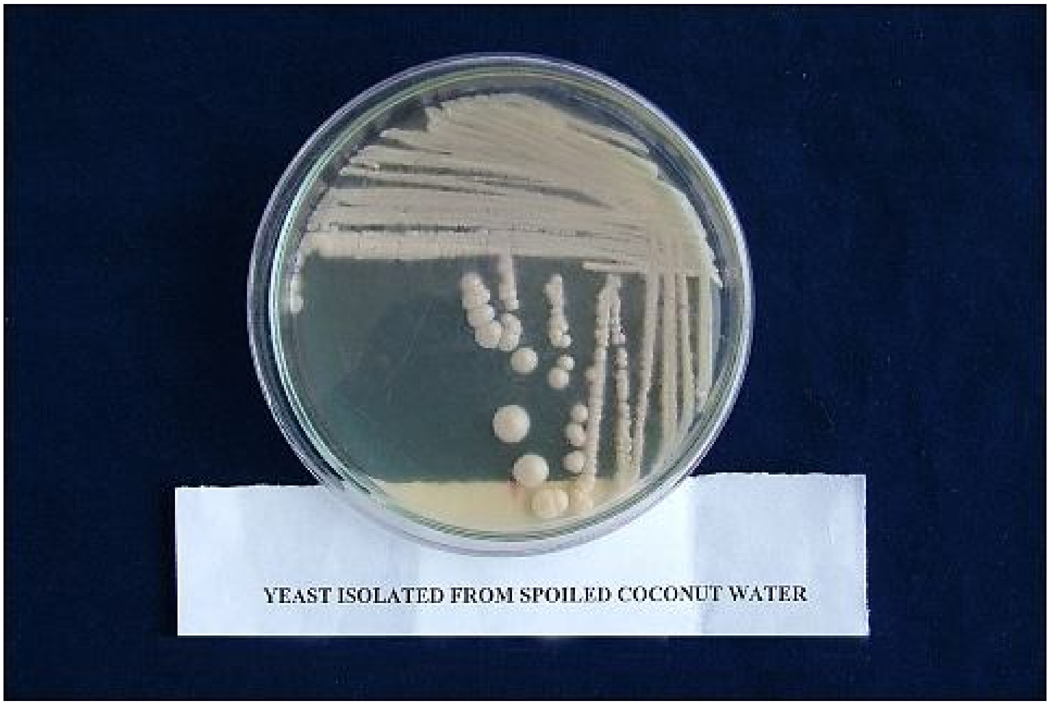
Fermentation of tomato pulp was carried out for 3 days and alcohol content was estimated at 24 h intervals. The data in Table (20, 21 & 22) and Fig. (7 & 8) show that the alcohol production was highest and around 80 g/lit by both yeast cultures. Alcohol content in treatments with both pectinase and amylase, and also with both enzymes and sugars was higher (59 to 65 g/L) with both cultures and they were on par. The alcohol content was at lowest in the treatment with substrate alone (16 g/L and 15 g/L) followed by the treatment where substrate is treated with pectinase (19.18 g/L and 17 g/L) with both cultures. However the alcohol levels were on par among above treatments indicates that addition of pectinase to tomato pulp did not help in improving alcohol production.

Addition of sugar to substrate gave alcohol yields (54 g/L and 55 g/L) with both cultures with enzymes and enzymes with sugar treatments gave significantly higher alcohol yields compared to addition of sugar alone to substrate (T6 & T13) wherein alcohol content was 54 g/L and 55 g/L. It is clearly evident from the data that the addition of amylase (T2 & T9) to substrate did give significantly higher alcohol content compared to substrate alone (T5 & T12). Addition of amylase along with pectinase to tomato pulp gave better alcohol yields. It is interesting to note that the situation is different in mango wherein addition of pectinase along with amylase did not help in improving in alcohol content but it was effective in case of tomato which could be possibly due to higher pectin content in tomato compared to pectin content in mango pulp. The data also indicate clearly that it is possible to improve fermentative production of alcohol by external addition of enzymes or by fermenting with yeasts having pectinolytic and amylolytic activity or by culturing with pectinolytic, amylolytic microbes along with alcohol producing yeasts. Banana peels were saccharified by using acid enzyme and steam for production of ethanol by fermentation (Tewari *et al.*, 1986).

When alcohol production was followed for three days alcohol content increased by about 10% in case of treatment with both enzymes pectinase and amylase while in treatment with sugar as substrate the alcohol content decreased by about 5% only. In other treatments, though alcohol content increased slightly by 48 h, it was reduced by 72 h. In substrate treated with pectinase alone showed slight increase in alcohol content by 48 h and remained constant for the rest of the period of fermentation.

The data on fermentative production of alcohol from tomato with different treatments during 3-day period shows that more than 90% of alcohol is produced by 24 h and hence alcohol production can be terminated after 24 h. Similar results are found with both standard culture MTCC-172 and local yeast isolate AM-113 indicating that either of the cultures can be used for alcohol production from tomato or mango pulp. However, the cultural conditions have to be standardized further such as inoculum percentage optimal temperature, co-culturing with pectinolytic and amylolytic cultures as required.

Since addition of enzymes helped in improving alcohol production from (23.41 g/L) to (64.50) in mango and (16.7 g/L) to (65.07 g/L) in tomato pulp respectively indicating (2.75) and (3.89) times increased production of alcohol.



Colony Morphology of Yeast Isolates AM-111

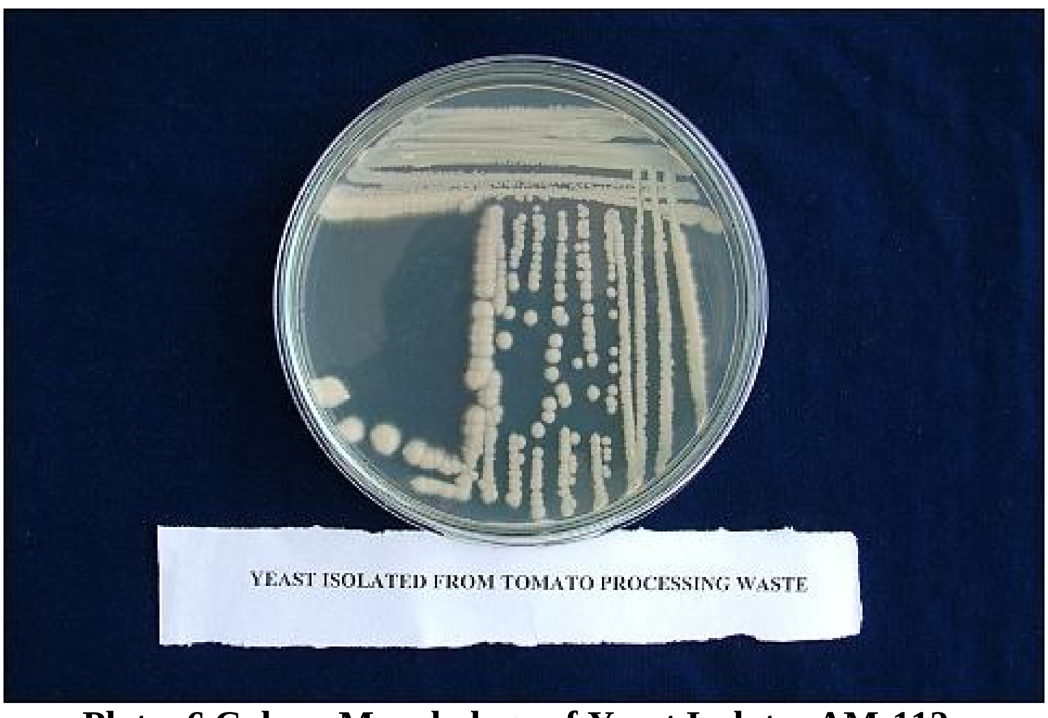


Plate: 6 Colony Morphology of Yeast Isolates AM-112

Colony Morphology of Yeast IsolateAM-114

Plate: 7 Colony Morphology of Yeast IsolateAM-115

Colony Morphology of Yeast IsolateAM-116

Plate: 8 Colony Morphology of Yeast IsolateAM-117

Plate: 5 Colony Morphology of Yeast IsolateAM-113

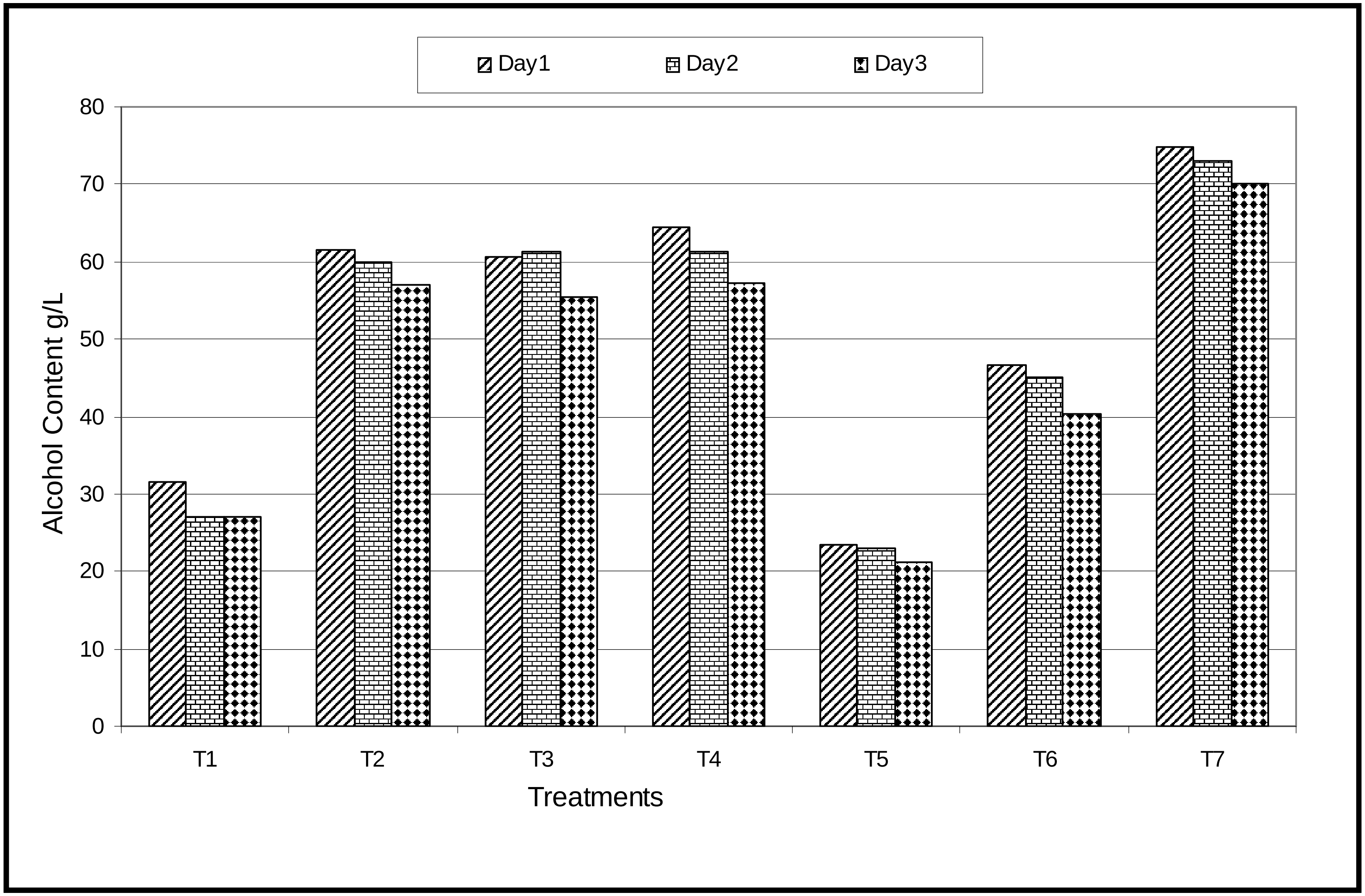


Fig: 1 Alcohol Content in Mango pulp after fermentation with *Saccharomyces cerevisiae* MTCC-172

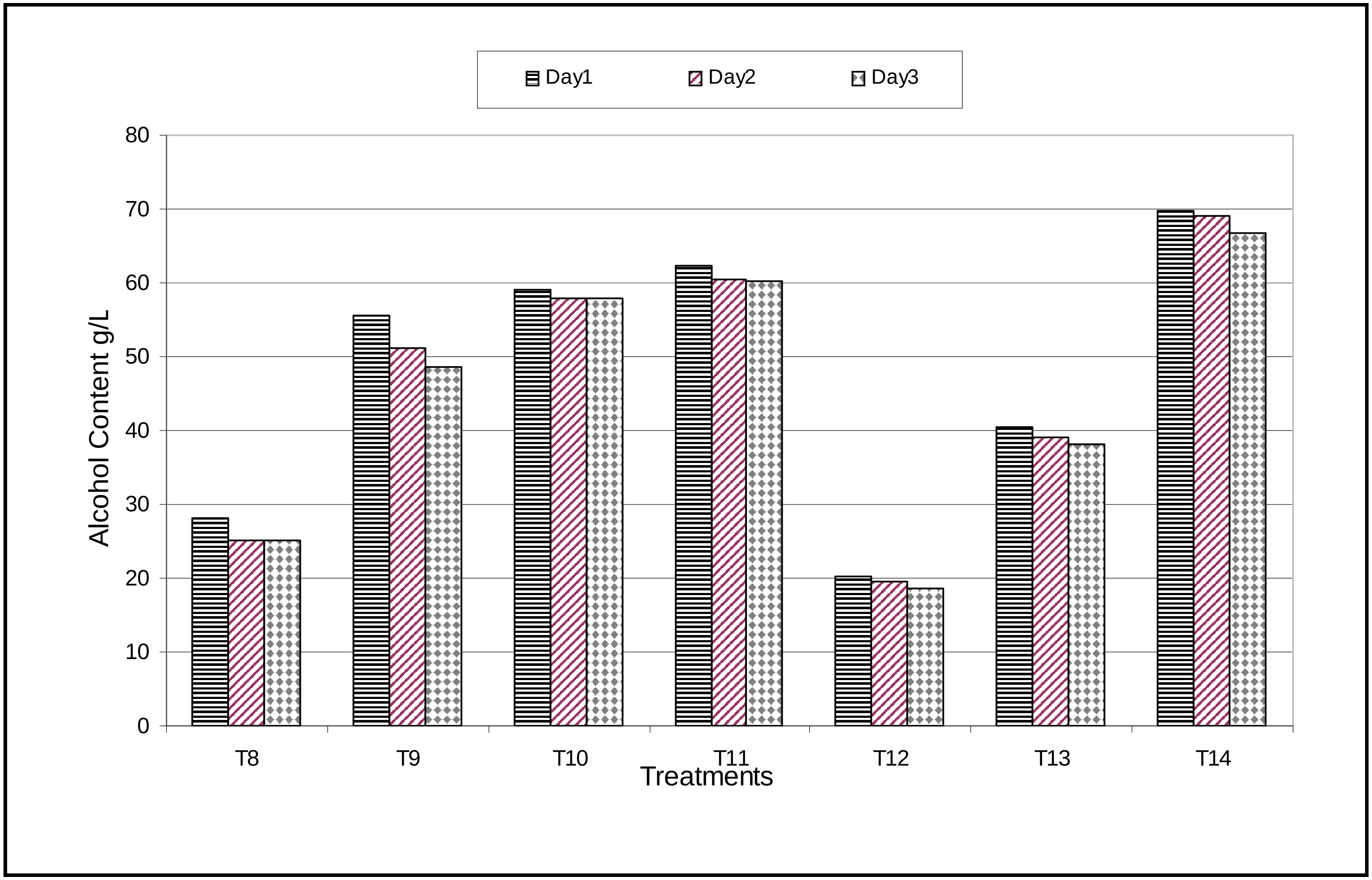


Fig: 2 Alcohol Content in Mango pulp after fermentation with yeast isolate AM-113

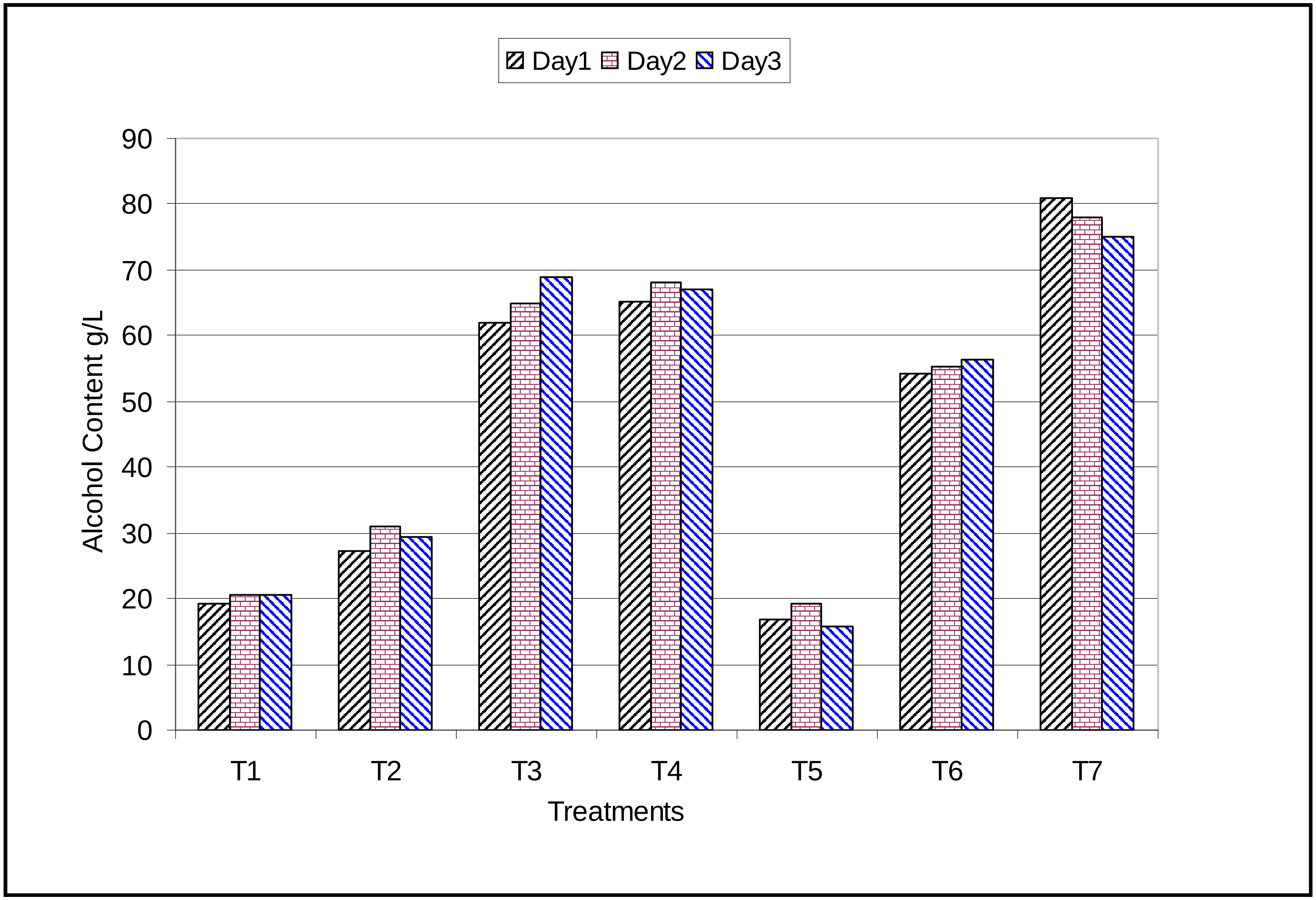


Fig: 7Alcohol Content in Tomato pulp after fermentation with *Saccharomyces cerevisiae* MTCC-172

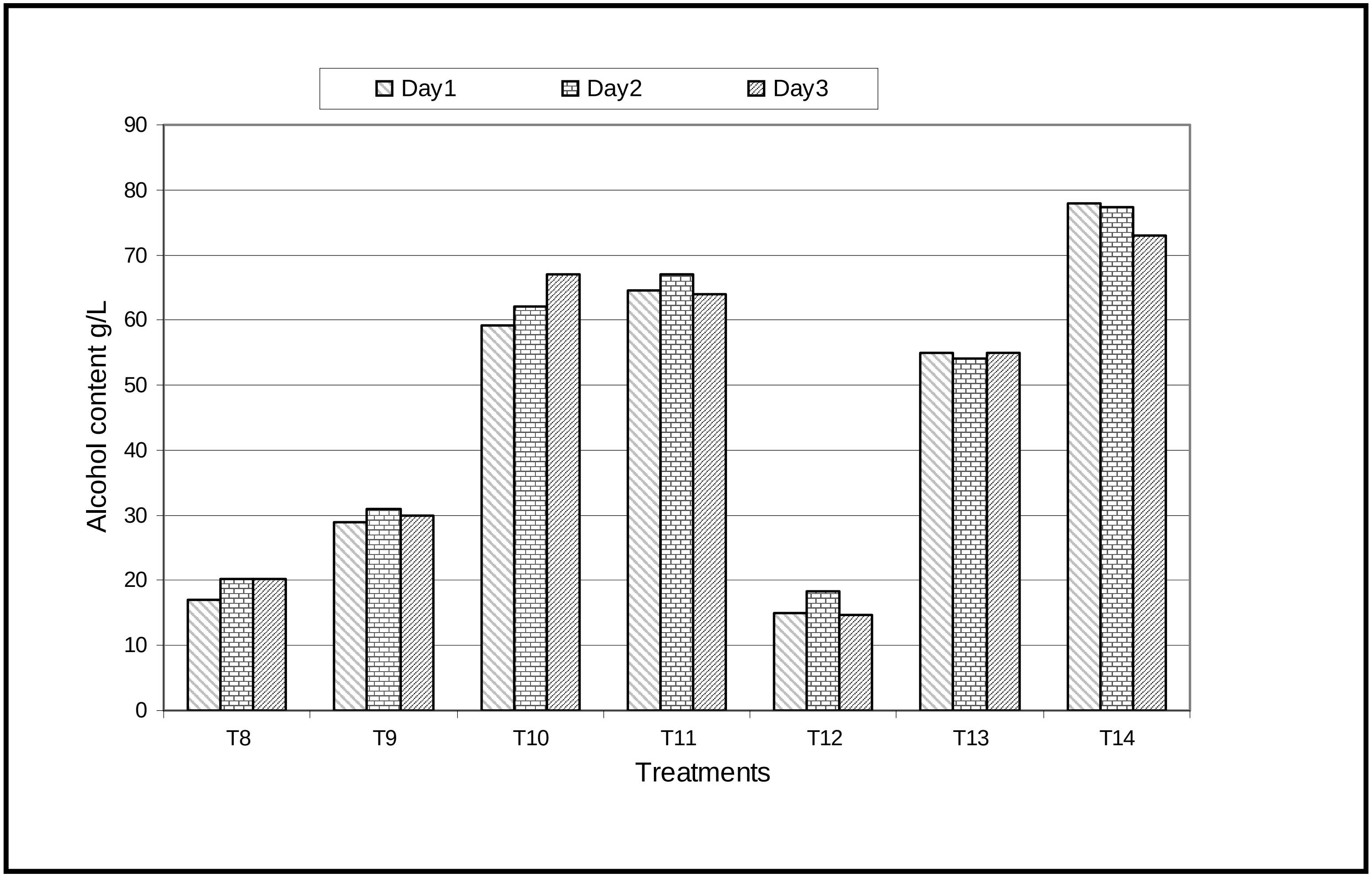


Fig: 8 Alcohol Content in Mango pulp after fermentation with yeast isolate AM-113

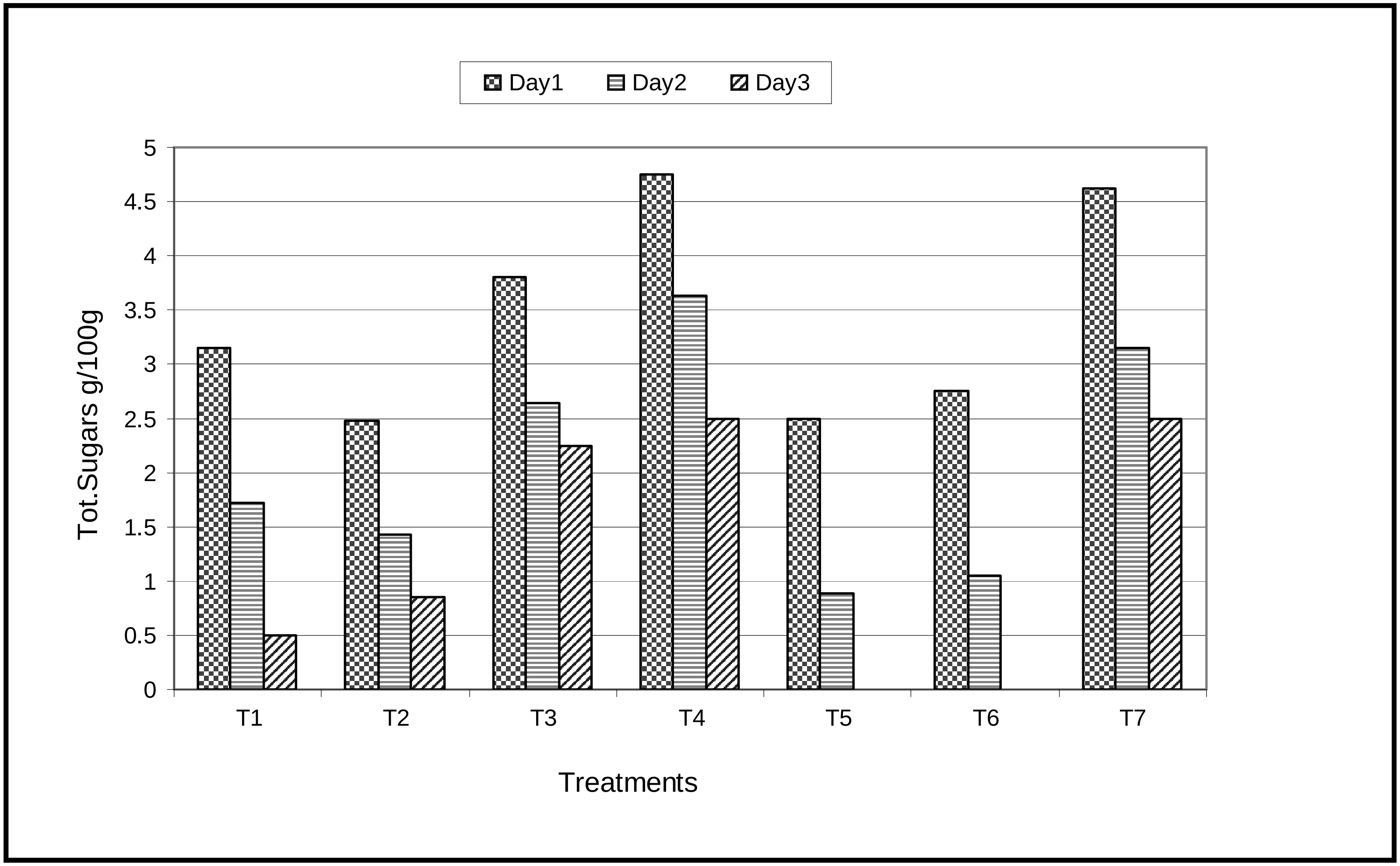


Fig: 5 Residual Total Sugars in Mango pulp after Fermentation with *Saccharomyces cerevisiae* MTCC-172

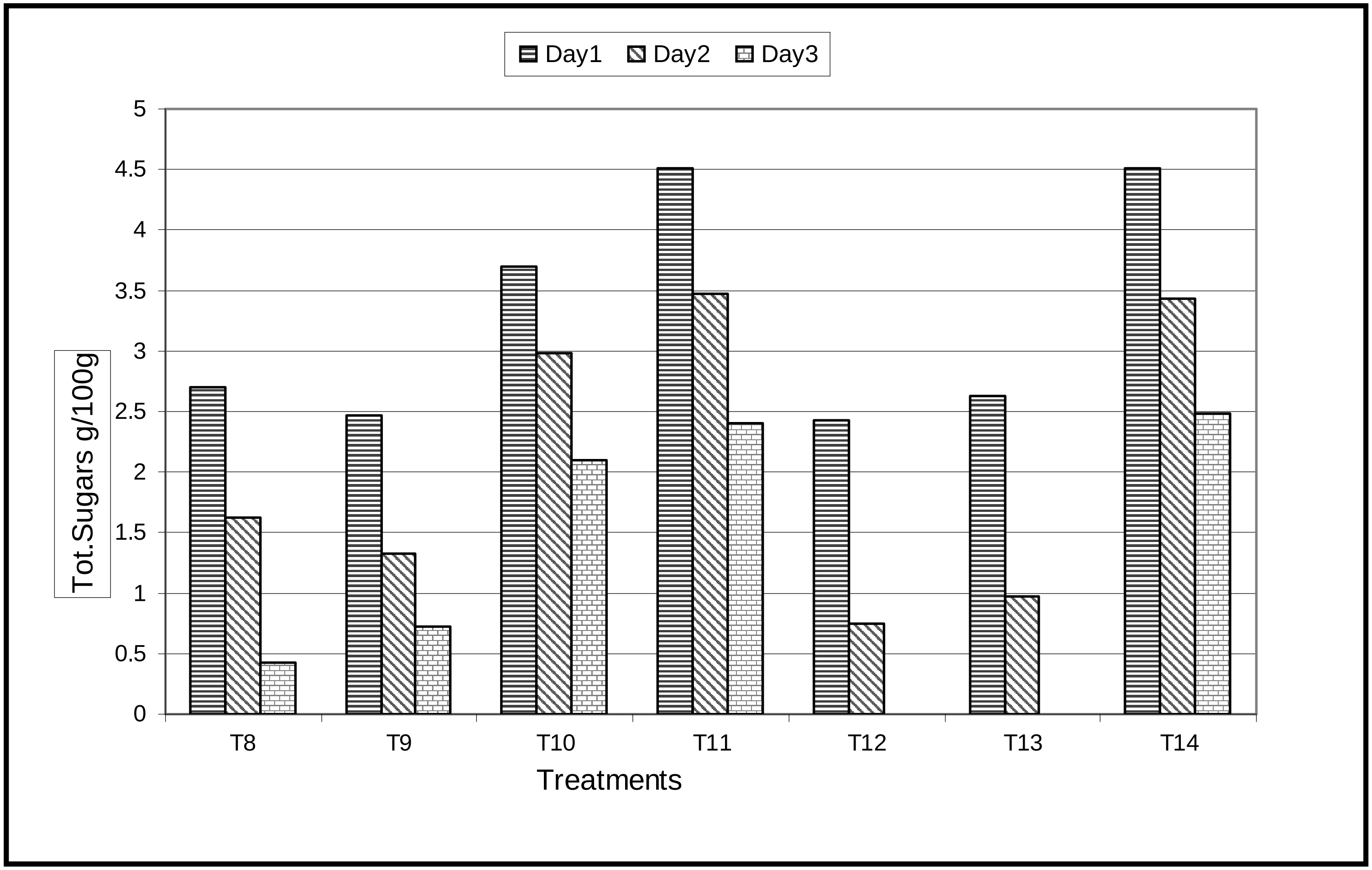


Fig: 6 Residual Total Sugars in Mango pulp after Fermentation with yeast Isolate AM-113

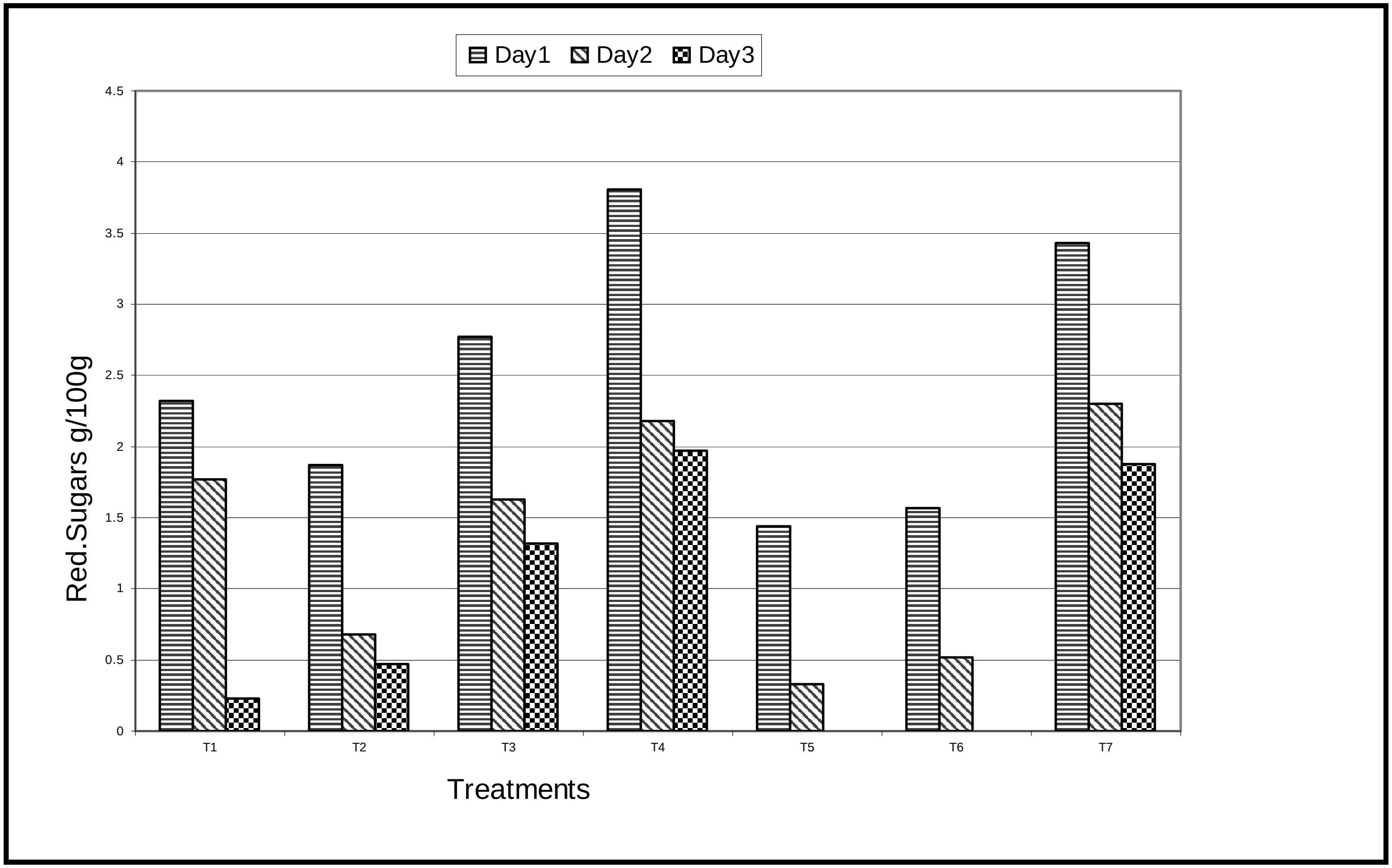


Fig: 3 Residual Reducing Sugars in Mango pulp After Fermentation with *Saccharomyces cerevisiae* MTCC-172

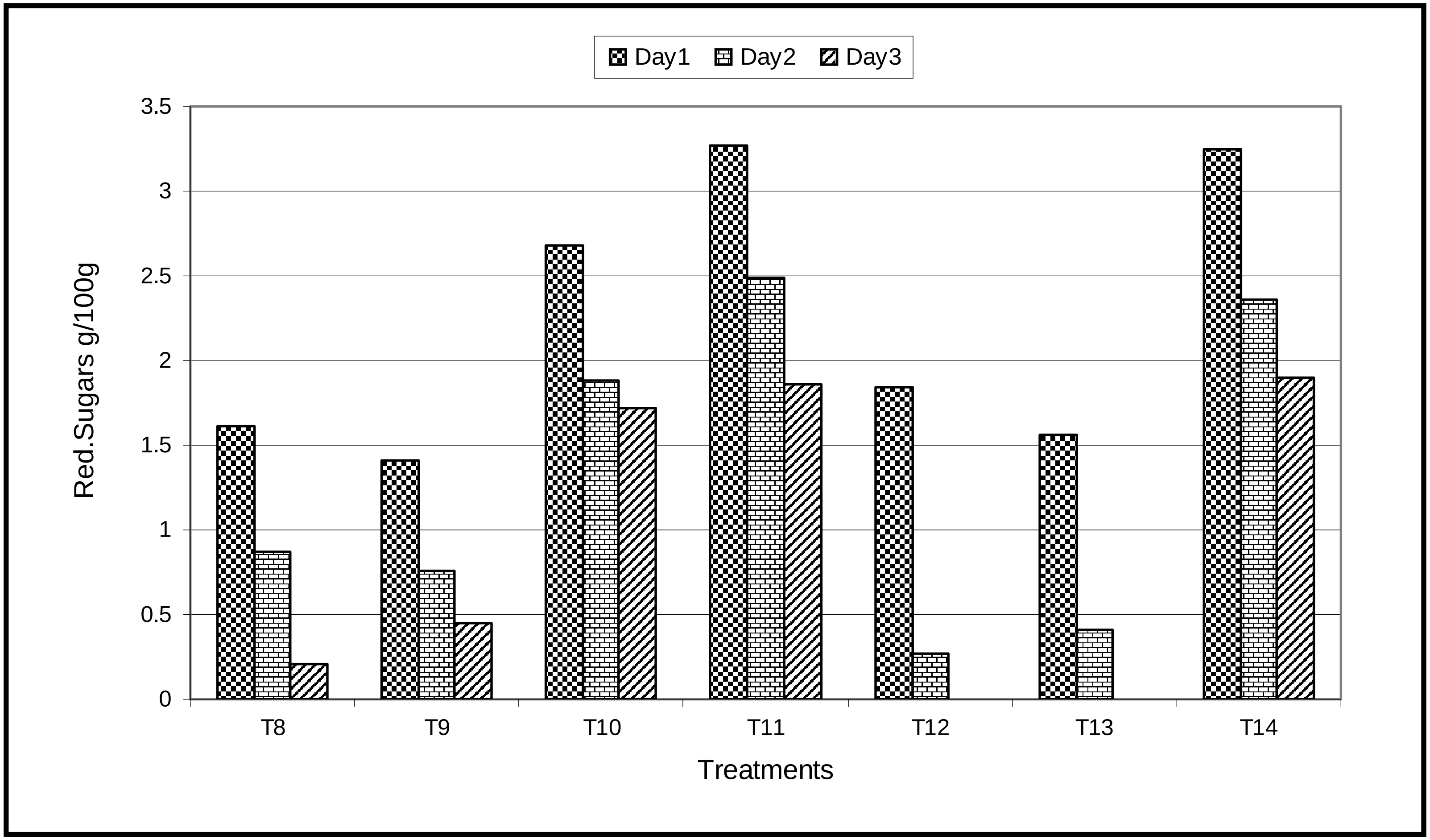


Fig: 4 Residual Reducing Sugars in Mango pulp after Fermentation with yeast isolate AM-113

CHAPTER – VI

SUMMARY

The present investigation was carried out with the objective of isolating pectinolytic and or amylolytic yeast cultures and fermentative production of alcohol from mango and tomato pulp can be summarized as:

1. A few yeast cultures AM-113 AM-115 and AM-117 showed slight or negligible pectinolytic activity, whereas other cultures did not show pectinolytic activity at all.
2. Yeast isolates from different sources did not show amylolytic activity
3. All the isolates had the ability to produce alcohol fermentatively. The maximum alcohol content was observed with *S. cerevisiae* MTCC-172 (80.00 g/L) whereas lowest alcohol content was with yeast isolate AM-111 (54.00 g/L).
4. Addition of pectinase @ 2% increased the flowability of the pulp in both mango and tomato.
5. Treatment of mango pulp with amylase @ 2% made it slightly viscous whereas in tomato it was free flowing after amylase treatment.
6. Treating the substrate with both the enzymes @ 2% increased the flowability and increase in brix in both mango and tomato pulp.
7. Addition of pectinase to mango and tomato pulp showed increased alcohol production 31.60 g/L and 19.18 g/L respectively; with mango and tomato pulp i.e. about 26% and 16% increase over substrate alone treatment.

8. Treatment of substrate with amylase had increased alcohol production (61.48 g/L) compared to substrate alone (23.41g/L) with mango pulp. However compared to mango, in tomato pulp, the increase was lesser (27.31g/L) in amylase treatment when compared to substrate alone (16.70 g/L). There was nearly more than 140% and 60% increase in alcohol production with mango and tomato pulp respectively.

9. Treating the substrate with both amylase and pectinase in mango pulp did not show any additional increase in alcohol (60.67 g/L) over amylase alone treatment (61.48 g/L), whereas in tomato pulp alcohol production was more than doubled (61.94 g/L) compared to amylase alone treatment (27.31 g/L).

10. Treating the substrate with both amylase and pectinase along with sugar did show a marginal increase of about 6% in alcohol content of 64.50 g/L and 65.07 g/L in mango and tomato pulp respectively compared to amylase and pectinase treated pulp.

11. Treating the substrate with sugar, the alcohol content nearly doubled in both mango and tomato pulp (46.71 g /L and 54.31 g /L) from (24.41 g/L and 16.70 g/L) respectively.

12. Substrate alone gave much lower amounts of alcohol 23.41 g/L and 16.70 g/L in both mango and tomato pulp when compared to substrates added with enzymes indicating the need for the use of enzymes for releasing more substrate for their further conversion to alcohol.

13. Finally, it can be concluded that the over ripened mango fruits and tomatoes can be used for fermentative production of alcohol effectively and economically.