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**RESEARCH ARTICLE**

# COMPARATIVE STUDIES OF L-LACTICACID PRODUCTION FROM GROUND NUT SHELL AND SUGARCANE MOLASSES BY MUTANT LACTOBACILLUS DELBRUECKIINCIM2025 U-25 STRAIN.

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## *Abstract*

The lactic acid production from groundnut shells is economical since the raw material is cheaply available source even though the yield of lactic acid is about 30g/lit. This would be a potential source for producing the lactic acid. However the lactic acid from other sources like sugarcane molasses is tried to enhance the yield. It is observed that lactic acid yield is 23.5g/lit from the sugar concentration of 20g/lit.Which is more than the strains without mutations, which is about 20g/lit. Mutants were generated by exposing to UV radiations, with different exposure times(5,10,15,20 and 25 min) and stability is tested on 0.6%Allyl aicohol.Among the mutants the results fromUV-25 strain were encouraging and the lactic acid produced was about 30 g/l from groundnut shells and 23.5g/lit from sugarcane molasses at temperature of $42^0$c&$40^0$C. The pH optimum was found to be about5.5 & 5.2 and inoculum size was found to be 5ml& 3 ml. The present comparative studies were focused on the concentration of lactic acid from groundnut shell and sugarcane molasses by mutant strain U-25.



## Introduction:-

Lactic acid is a monomer which is used to produce a biopolymer PLA (Polylactic Acid), and PLA can be a good alternative to the polymer produced by petrochemical route(Rojan et.al.,2009). Lactic acid demand is expected to increase due to the development of new, large-volume uses, particularly as a feedstock for biodegradable polylactic acid (PLA) polymers, oxygenated chemicals (Datta and Henry, 2006).Most of the lacto bacilli and Lactococci were found to ferment xylose(Colliansand James ,1984) . The yield coefficient of lactic acid was reported by (Tanaka and komiyama2002) , and found that it exceeded 1mol/mol in cultivations when initial xylose concentrations were more than 50 g/l. However the fermentative rate of xylose by pentose –fermentative bacteria is lower than that of glucose by homo fermentative lactic acid bacteria such as L.casei,(Garde et.al(2002) · A mathematical model to simulate simultaneous saccharification and lactic acid fermentation was proposed by( Luo et.al 1997). It is very expensive when sugars, e.g., glucose, sucrose, starch, etc., are used as the feedstock for lactic acid production. Therefore, lignocellulosic biomass is a promising feedstock for lactic acid production considering its great availability, sustainability, and low cost compared to refined sugars. Despite these advantages, the commercial use of lignocellulose for lactic acid production is still problematic. This review describes the "conventional" processes for producing lactic acid from lignocellulosic materials with lactic acid bacteria. These processes include: pretreatment of the biomass, enzyme hydrolysis to obtain fermentable sugars, fermentation technologies, and separation and purification of lactic acid.(Mohammad Ali et.al 2011). Both starchy and lignocellulosic biomass have been extensively used, however lignocellulosic biomass in Ghana is generated in large volumes as crop residues and mostly considered waste although some amount is used as animal feed. These crop residues are readily available as cheap raw materials for lactic acid production. By employing appropriate fermentation processes about 199,856

tonnes, 244,305 tonnes, 127,715 tonnes and 362,003 tonnes of lactic acid at 50 % utilization can be generated from maize cobs, millet stalk, sorghum stalk and rice straw respectively for the international market(Richard, 2015). Pure substrstes such as glucose(KWON,2001)or lactose(Amrana.a.,1996)commonly used for the production of lacticacid.The starch (Altaf,2006)and cellulose were economically unfavourable because they require expensive pretreatment procedures in order to release fermentable sugars. The production cost of lactic acid can be significantly reduced if the by-product of sugar industry ,sugar cane molasses(40 to 60% sucrose)which can be efficiently converted into lacticacid by lactobacillus species(Dumbrepatil,Mukund Adsul,2008).

In the present research, ground nut shells and sugarcane molasses was used as carbon substrate, since India is one of the largest producers of ground nut and sugarcane. There are no significant reports on accumulation of lactic acid above 20 g/l with ground nut shells as substrate. we focuses on the pretreatment of raw material ie., ground nut shells and optimizing the temperature conditions for Lactic acid production by mutant strain, of Lactobacilli debruekii U-25was applied . It was observed during the experimental studies that at $42^0$C the maximum yield of lactic acid with mutant strainUV-12(1) about 23g/l.(C.Obula Reddy, AVNSwamy,2015) and from sugarcane molasses lacticacid yield is about 18g/lit.

## Materials and methods:-

**Materials:-**

The chemicals from Hi-Media Limited, Mumbai, India, are used during the present experimental studies. The other chemicals like Lactic acid, yeast extract, peptone, and tween-80 are obtained from S.D. Fine Chemicals Limited, Mumbai, India. The lingo cellulose waste materials used in the present studies are made from ground nut shell, taken from local market.

**Molasses:-**

This was obtained from Jeypore sugar company, Chagallu, West Godavari Dist ,Which was hydrolyzed to fermentable sugars.

**Micro-organisms and growth media:-**

The micro organism L. delbrueckii NCIM 2025 was selected for study and was grown in MRS media. The strain was procured from National Collection of Industrial Microorganisms (NCIM )NCL,pune and was maintained at $4^0$C.

**Inoculum preparation:-**

Lactobacillus delbrueckii strain is improved by exposure to UV lamp radiation (254nm, 30W) to inhibit adh activity. Log phase cells of Lactobacillus delbruekii are harvested by centrifugation at 5000 rpm for 10 minutes and are further processed aseptically.

Lactobacillus delbrueckii cells were transferred from stock cultures and are grown to freshly prepared agar plates. After incubation at $35^0$C for 24 h the cells are transferred to 100 mL sterile growth medium in 50 mL screw cap tubes for inoculums preparation. In 100 mL MRS media 5 mL of this culture are inoculated for growth of cells and left for 24 h.

**Mutagenesis:-**

Actively growing cells (10 mL) from the log phase is harvested by centrifugation (Elico. Hyderabad) at 5000 rpm for 10 min. The supernatant is decanted, and cell pellet is washed with 0.9% NaCl. The washed pellets are re-suspended in 10 mL 0.9% NaCl where total viable count is found (2 X $10^6$ cells / mL). Ten milliliter of the diluted cell suspension was irradiated with the UV lamp (254 nm) at 20 cm distance and the samples are taken after 5, 10,15, 20 and 25min. The samples are serially diluted in sterile saline solution and survivors are determined by streaking 0.1 mL of the diluted sample on a agar medium containing cane sugar 10 %, yeast extract 1 %, CaCO3 0.5 % and agar 2 %. The viable count is determined after incubation at $42^0$C under vacuum till the distinct colonies starts appearing.

**Selection of mutants:-**

The irradiated cells are plated on to fermentation medium and colonies are subsequently transferred to agar plates containing 60 mL/L Allyl alcohol as selection agent. This compound inhibits parent type cells since cells with

functional alcohol dehydrogenase activity converts allyl alcohol to the toxic compound acrolein which kills the cells and thus only adh –ve cells are selected. Hence it was inferred that the cells that were selected were mutants.

**Pretreatment of ground nut shell (GS):-**

The ground nut shell is taken and washed with water to remove any contaminants. Later dried at $45^0$C in a hot air oven(Biotechnics, Hyderabad)) . After drying ground nut shell was chopped into small pieces. Small pieces then grounded or milled in electric grinder to attain the size of 0.5mm. The 10 gm of biomass of ground nut shell was taken in a conical flask and treated with different concentrations of following chemicals;

1. Sodium sulfite pre treatment method; 10gm of GS was treated with 100ml of 10%, 15% and 20% concentrations of Na2SO3. All the three were sterilized in an autoclave at 121oc for 15 min. After autoclaving contents were filtered through two layers of muslin cloth. Solid residue was repeatedly washed with distilled water until the Ph of the filtrate become neutral. The residue was dried at $45^0$C for overnight.
2. NaoH pre treatment method; 0.25N, 0.5N, 1N AND 1.5N concentrations of NaoH is used to pre treat the GS as mentioned above.
3. Dilute acid hydrolysis method; 10 gms of GS is treated with 100ml of 0.25N, 0.5N and 1NHcl.Repeat the steps as mentioned above.
4. Finally treat the 10gms of GS with 0.25N, 0.5N and 1N concentrations of $H_2SO_4$.
5. Organic solvent extraction method; 10 grams of GS taken in a thimble of soxhlet extractor(Borosil, Mumbai).100 ml of methanol is taken in a round bottom flask. Solvent extraction is carried out at $100^0$C.condensed vapors percolate through GS and biomass is softened.

**Estimation of sugars- After pretreatment sugars are estimated from GS by 3, 5-DNS method:-**

Submerged fermentation;-sugarcane molasses is used as fermentation media. Fermentation media is prepared by mixing sugarcane molasses with production media.

**Table 1:-** chemical composition of cane molasses

| Ingredients | %Dry weight |
|---|---|
| Water | 20 |
| Sugar content | 62 |
| Ash | 8 |
| Non sugar contents | 10 |

## Analytical methods:-

**Cell mass analysis:-**

Cell mass analysis is done after the centrifugation at 10,000 rpm for five min and thus cells obtained as pellet are washed three times with distilled water and then undergo vacuum suction dry for 24 hours for determination of final weight. The dried cells are dissolved again in fixed volume of double distilled water and then diluted in different range of concentration. The growth of the cells and the fermentation product are measured at 0, 4, 8, 12, 24 and 48 hours from slope of standard plot.

**Lactic acid analysis:-**

Total lactic acid is determined by colorimetric method by UV-VIS spectrophotometer (ELICO SL 164 double beam) by using p-phenyl phenol.

**Sugar analysis:-**

The total sugar was determined by the phenol-sulphuric method and reducing sugar was determined by the DNS (Di-nitrosalisylic) method while sugar glucose was calculated according to the method describe by Dubois et al.

**Gas Chromatography:-**

The concentration of ethanol, was determined by a gas chromatograph (AIMIL, Nucon, India, Series 5765) equipped with Chromosorb 101 column using nitrogen as the carrier gas and a mixture of hydrogen and oxygen gas to sustain the flame. The detector, injector and oven temperature were maintained at $200^0$C, $195^0$C and $180^0$C respectively. A gas flow rate of 35 ml/min was maintained.

## Results and discussion:-

**Mutagenesis and selection of mutant:-**

The mutant of L. delbrueckii is developed after irradiation of UV rays at different time intervals. Without UV, total 130 colonies were obtained in agar-agar plate (20 g/L). After UV exposure (30 W, 245.7 nm, 20 cm, for 5, 10,15,20 and 25min) 54, 25, 8, 4&2 colonies are obtained respectively and are grown in plate containing 60 mL/L Allyl alcohol(Fig-1). Colonies survived on Allyl alcohol plate are grown repeatedly (four times) on 60 mL/L Allyl alcohol plates. The mutant colonies are named as U-5 for colonies after 5 min UV treatment. Colonies obtained after five min exposure (U5-1 to U5-2) when transferred to agar plate containing 60 mL/L Allyl alcohol, colonies are not survived. Colonies obtained after 10 min exposure colonies are not survived. These colonies are transferred three times successively on Allyl alcohol to check their stability. U-10 is unable to grow after third transfer. Similar treatment is done for strain no. U-20 and U-25 found that U-25 is stable for more than four generations. Thus, finally U-25 was selected for study in batch fermentation for cell mass sugar utilization and Lacticacid production profile.

**Table 2:-** selection of mutant strain

Mutants are selected given in the following Table.

| SNO | Exposure Time(min) | No of colonies | Stability on 0.6% allyl alcohol |
|---|---|---|---|
| 1 | 5 | 53 | 0 |
| 2 | 10 | 28 | 0 |
| 3 | 15 | 8 | 0 |
| 4 | 20 | 4 | 0 |
| 5 | 25 | 2 | yes |

**Fig 1:-** colonies after UV –Mutagenesis.

Dilute acid hydrolysis of GS ;The results of the GS are given in the following table

**Table.3:-** Acid hydrolysis of Ground nut shell.

| **HCL(conc.)** | **Amount of sugars released (gm/lit)** | **H2So4(conc.)** | **Amount of sugars released (gm/lit)** |
|---|---|---|---|
| 025N | 27 | 0.25N | 30 |
| 0.5N | 46.5N | 0.5N | 57.5 |
| 1N | 43.5 | 1N | 48.2 |

Though four methods of saccharification of GS tried, only acid hydrolysis by dilute H2S04gave good result(Table.3)

**Fermentation profile of parent and mutant strain in MRS media:-**

The batch fermentation kinetics of the parent type strain of L. delbrueckii NCIM 2025 is studied at $40^0$C and pH 5-6 under semi-anaerobic conditions with initial ground nut shells. The fermentation profiles of biomass, glucose, lactic acid are shown in Fig. 1. Lactic acid is produced up to 22 g/l during the exponential growth of the cells and in 48 hrs lactic acid concentration reaches to a final concentration . After 48 hr, the lactic acid production rate is almost constant. The pH is maintained at 5.5 by addition of 2 mol/L KOH.

The mutant cell U-25 is inoculated in MRS media for lactic acid production shows good growth and lactic acid production. The lactic acid concentration was 30g/l (0.97 gLA/sugar consumed) and cell mass concentration is 13±1.5 g/L, while sugar utilization is from 85 % to 89 % of total sugar.

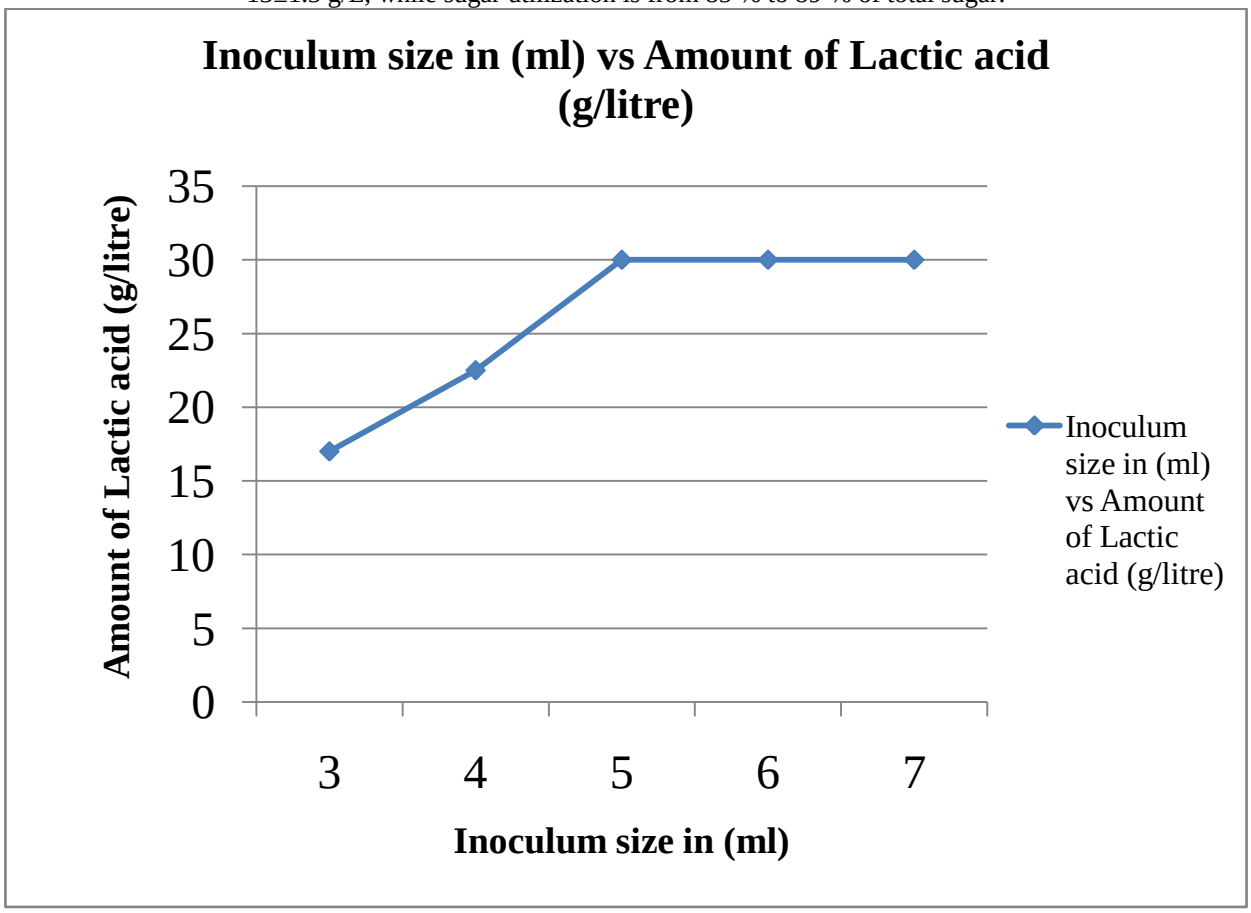


**Fig 2:-** Effect of inoculum size on lactic acid production.

The effect of inoculum size on lactic acid accumulation is studied in the present studies. It is observed that initially the lactic acid accumulation is about 30 g/l at 5 ml inoculum size and is more or less constant at 7 ml. this may be attributed to optimum inoculum size at steady state conditions.

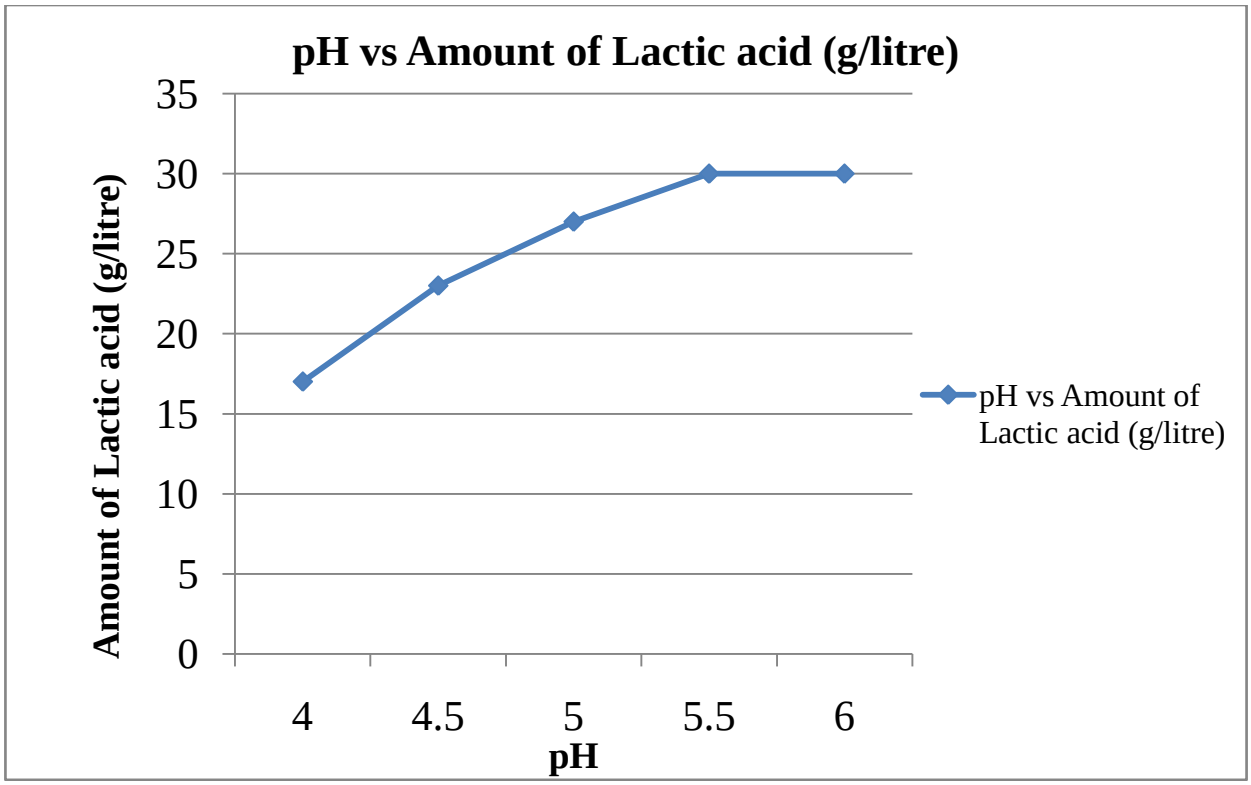


**Fig.3:-** Effect of pH on lactic acid production.

The effect of pH on lactic acid accumulation is studied and it is observed that about 5.5 pH the accumulation is about 30g/l. The maximum accumulation is due to attaining of steady state condition at this pH.

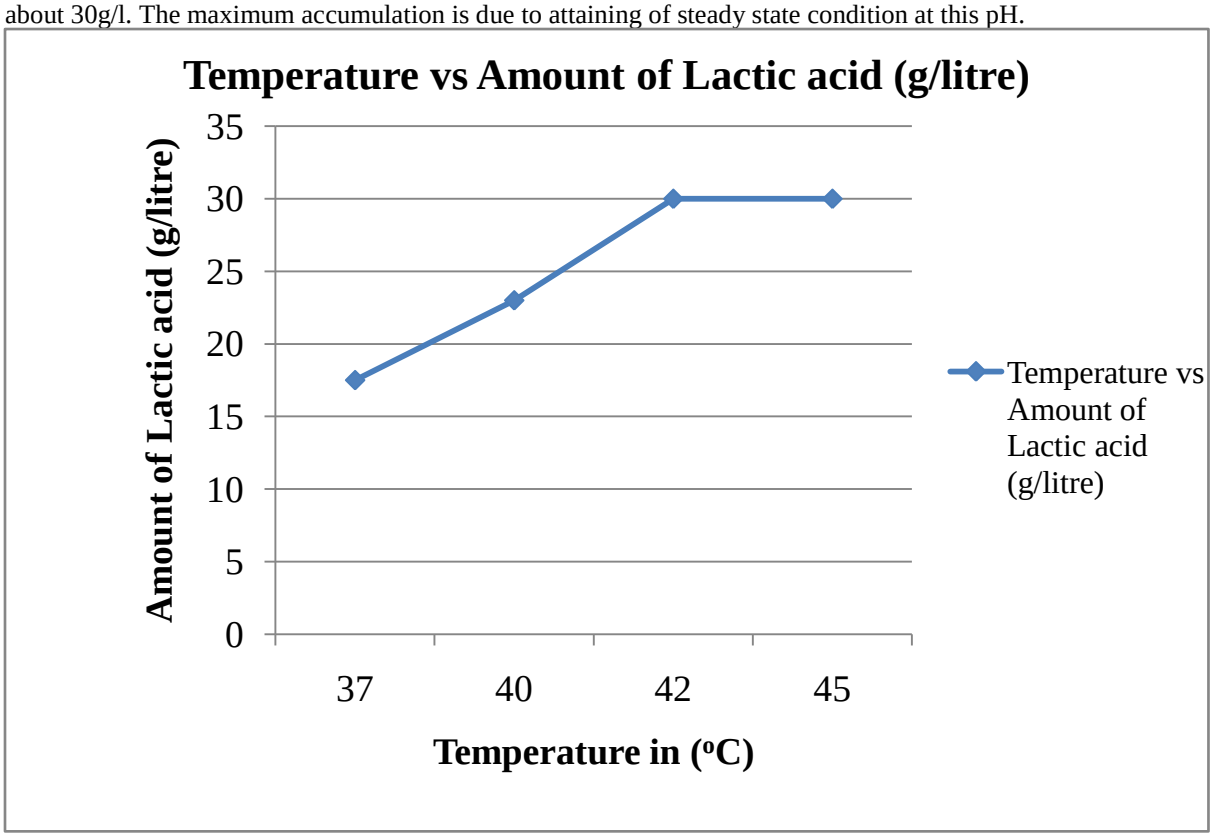


**Fig.4:-** Effect of Temperature on lactic acid production.

The effect of temperature on lactic acid accumulation is studied. The lactic acid accumulation is about 30 g/l at about $42^0$C.

**Submerged fermentation Comparison:-**
By optimizing the process parameters like substrate concentration, temperature, pH and inoculum size, the amount of lactic acid produced from sugarcane molasses by mutant strain is compared.

The following table shows the amount of lactic acid produced at different process parameters.
**Table 4:-** Amount of lactic acid produced at different process parameters for SF.

| S.No | Substrate conc. (g/l) | Temp. ($^o$c) | pH | I.S (ml) | Lactic acid (g/l) |
|---|---|---|---|---|---|
| 1 | 60 | 42 | 5.1 | 5 | 18 |
| 2 | 40 | 40 | 5.2 | 3 | 23.5 |
| 3 | 30 | 37 | 5.4 | 4 | 17.5 |

The maximum Lacticacid production from Groundnut shells was 23g/l when strain was exposed to 12 minutes of UV radiation(OBULA REDDY.C.,AVN.SWAMY,2016).In the present comparative study, The maximum Lacticacid with sugarcane molasses as substrate is 23.5g/l at 40hrs of fermentation time,where as the maximum Lacticacid is 30g/l with Groundnut shell as substrate with UV-25 strain(Fig-5).

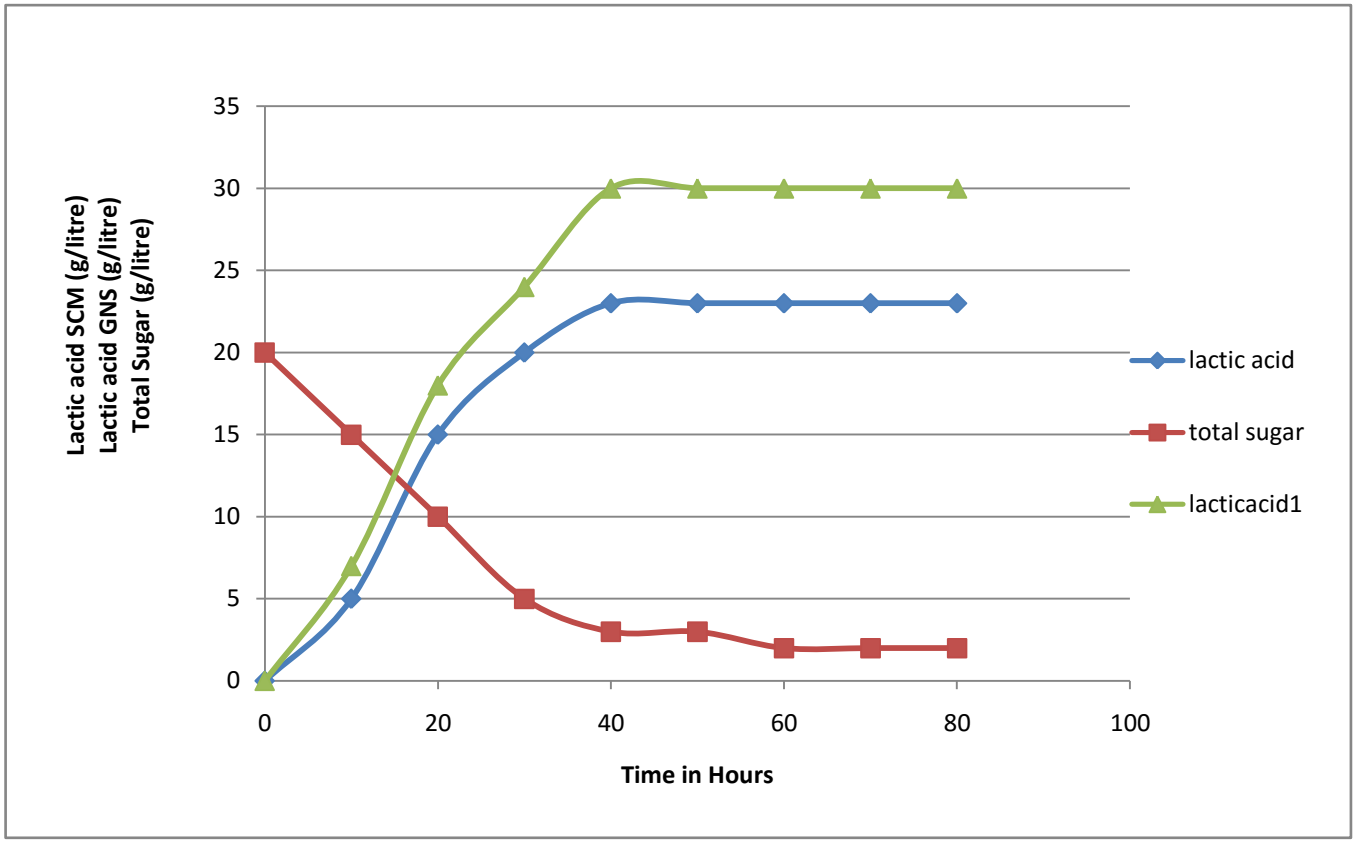


**Figure5: Lactic acid and Total sugar variation with fermentation time**

Lacticacid from sugar cane molasses (SCM)
Lacticacid(1) from Ground Nut Shell (GNS)
Total Sugar (TS)
In the present work,Lactobacillus delbrueckii mutant strain UV-25 was used to utilize lignocellulosic waste material such as Ground nut shell and agro industrial by product cane molasses for Lacticacid production by solidstate and

submerged fermentation. Lacticacid concentration was found to be 30g/lit from Ground nut shell, which were higher than in previous studies(OBULA REDDY.,SWAMY.AVN ,2016)and from 23.5 g/lit from 20gms of sugar from cane molasses at 40hrs of fermentation time.

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