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RESEARCH ARTICLE

EXPERIMENTAL STUDIES ON L- LACTIC ACID FROM GROUNDNUT SHELLS BY USING LACTOBACILLUS delbrueckii NCIM 2025 AFTER PRE TREATMENT.

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Abstract

In pursuit of selecting cheap raw material for producing lactic acid by fermentation ground nut shells were chosen as substrate. However there is no much relevant information was available on fermentation of ground nut shells. The experimental studies on production of L lactic acid were explored, by solid state fermentation. The experiments on groundnut shells were carried out, as substrate which was one of the cheapest raw materials available in India. The ground nut shells were pretreated with dilute acid (HCl&H_2SO_4), sodium sulphite, NaOH and methanol solvent extraction methods for producing total sugar. It was observed at temperatures ie., at 35^0C,37^0C and 42^0C, different inoculums size(3ml to 6ml) and different pHs(4.5 to 5.5), the lactic acid production is 12 g/l, `17 g/l and 23.0 g/l respectively. Lactic acid was estimated by AC Kimberley Taylor method. The mutant cells were tested for its stability on 0.6% Allyl alcohol , which was a selection agent. Mutants were generated by exposing to UV radiations, with different exposure times and stability is tested. The results were encouraging and the lactic acid produced was about 23 g/l at temperature of 42^0C. The pH optimum was found to be about 5.2. and inoculum size was found to be 3 ml. The present studies were focused on accumulation of lactic acid by using ground nut shells having ligno cellulosic material after treatment with dilute acid.

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). A novel research is done for first time, the recently developed proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS) apparatus as a rapid method for the monitoring of lactic acid fermentation (LAF) of milk. PTR-TOF-MS has been proposed as a very fast, highly sensitive and versatile technique but there have been no reports of its application to dynamic biochemical processes with relevance to the food industry.

Some researchers showed that PTR-TOF-MS is a powerful method for the monitoring of major volatile organic chemicals (VOCs) formed or depleted during LAF, including acetaldehyde, diacetyl, acetoin and 2-propanone, and it also provides information about the evolution of minor VOCs such as acetic acid, 2,3-pentanedione, ethanol, and off-flavor related VOCs such as dimethyl sulfide and furfural. This can be very important considering that the conventional measurement of pH decrease during LAF is often ineffective due to the reduced response of pH electrodes resulting from the formation of protein sediment (Christos, 2010).

In the present research, ground nut shells was used as carbon substrate, since India is one of the largest producers of ground nut . There are no significant reports on accumulation of lactic acid above 20 g/l with ground nut shells as substrate. There are various methods of optimizations but for factors having more than or equal to six variables, Taguchi methodology is assumed to be better since it simultaneously allow interaction studies. Lactic acid production parameter optimization using Lactobacillus amylovorus NRRL B-4542 was performed by Nagarjun et. al(2005) using the design of experiments (DOE) based on Taguchi protocol. They concluded that physical parameters like, temperature has higher influence, and among media components, yeast extract, MgSO4• 7H20, and Tween 80 play important roles in the conversion of starch to lactic acid and at optimum conditions lactic acid production was 93.50 (g/L). More recently lactic acid production from glucose by immobilized cells of Lactococcus lactis IO-1 was investigated using Taguchi methodology . The present studies focuses on the pretreatment of raw material ie., ground nut shells and optimizing the temperature conditions. Taguchi method for optimization of medium component for lactic acid production by mutant strain, of Lactobacilli debruekii was applied . It was observed during the experimental studies that at 42^0C the maximum yield of lactic acid of 23g/l.(C.Obula Reddy, AVNSwamy,2015).

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, tween-80 are obtained from S.D. Fine Chemicals Limited, Mumbai, India. The ligno cellulose waste materials used in the present studies are made from ground nut shell , taken from local market.

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 4^0C.

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 35^0C 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, 7, 8, 10 and 12 min. 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 42^0C 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 45^0C 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 121^oc for 15 min. After autoclaving contents were filtered through two layers of muslin cloth. Solid residue was repeatedly washed with distilled water until the $P^H$of the filtrate become neutral. The residue was dried at 45^0C 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.
   Finally treat the 10gms of GS with 0.25N, 0.5N and 1N concentrations of H_2SO_4.
4. 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 100^0C.condensed vapors percolate through GS and biomass is softened.
5. Estimation of sugars- After pretreatment sugars are estimated from GS by 3, 5-DNS method.

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 200^0C, 195^0C and 180^0C 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, and 5, 7, 8 10 and 12 min) 54, 32, 11, 4 and 2 colonies are obtained respectively and are grown in plate containing 60 mL/L Allyl alcohol. Colonies survived on Allyl alcohol plate are grown repeatedly (four times) on 60 mL/L Allyl alcohol plates. The mutant colonies are named as U8-1, U8-2 for colony no.1 & 2 after 8 min UV treatment. Colonies obtained after eight min exposure (U8-1 to U8-2) when transferred to agar plate containing 60 mL/L Allyl alcohol, but none are survived. Colonies obtained after 10 min exposure (U10-1, U10-2, U10-3, and U10-4), only U10-4 is survived. These colonies are transferred three times successively on Allyl alcohol to check their stability. U10-4 is unable to grow after third transfer. Similar treatment is done for colony no. U12-1 and found that it is stable for more than four generations. Thus, finally U12-1 was selected for study in batch fermentation for lactic acid, cell mass and sugar utilization profile.

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

Table.1:- 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	46.5	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.1)

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 40^0C 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 U12-1 is inoculated in MRS media for lactic acid production shows good growth and lactic acid production. The lactic acid concentration was 20g/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.

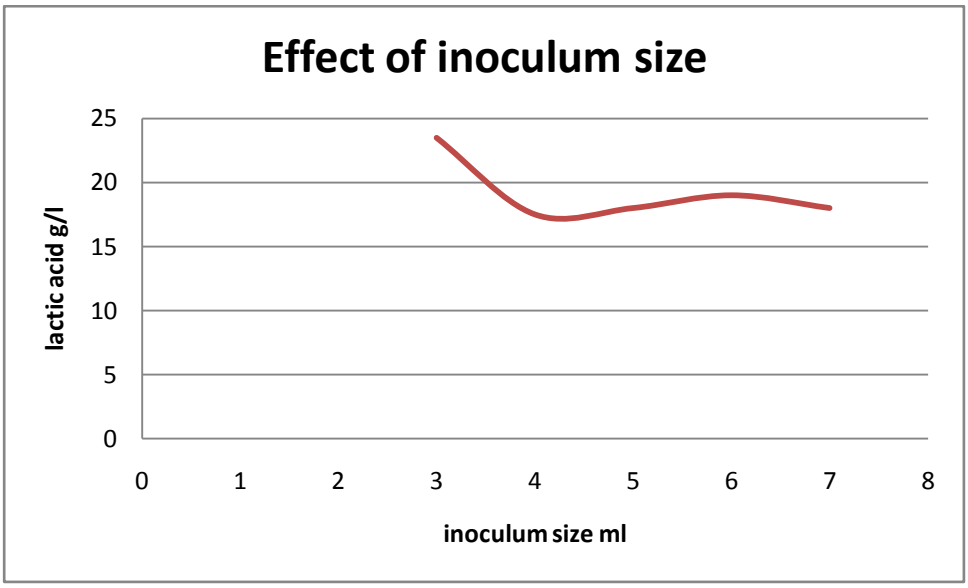

Fig:1:- Effect of inoculum size on lactic acid production.

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 23 g/l at 3 ml inoculum size and is more or less constant at 5 to 7 ml. this may be attributed to optimum inoculum size at steady state conditions.

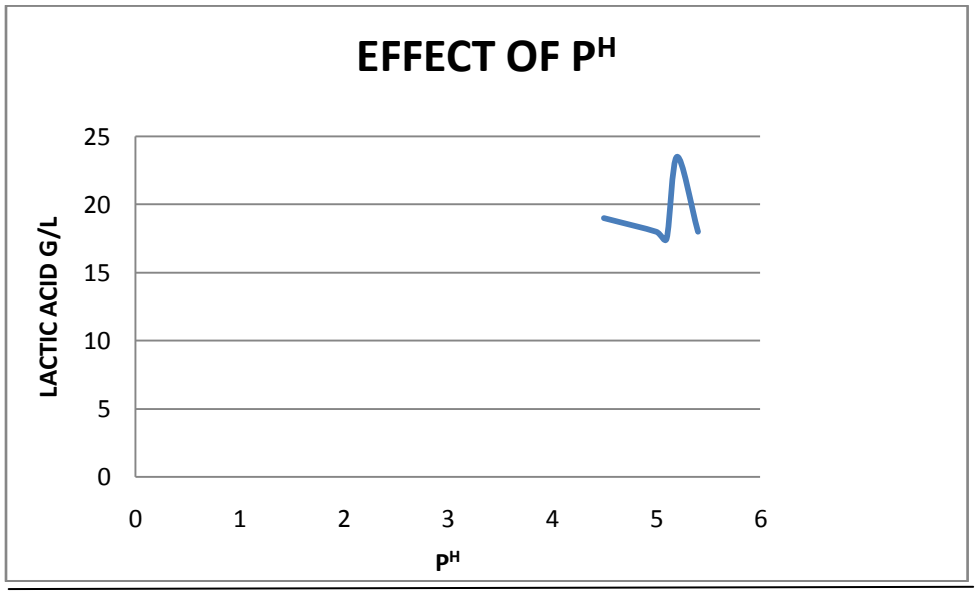

Fig.2:- Effect of pH on lactic acid production.

The effect of pH on lactic acid accumulation is studied and it is observed that about 5.2 pH the accumulation is about 23 g/l. The maximum accumulation is due to attaining of steady state condition at this pH.

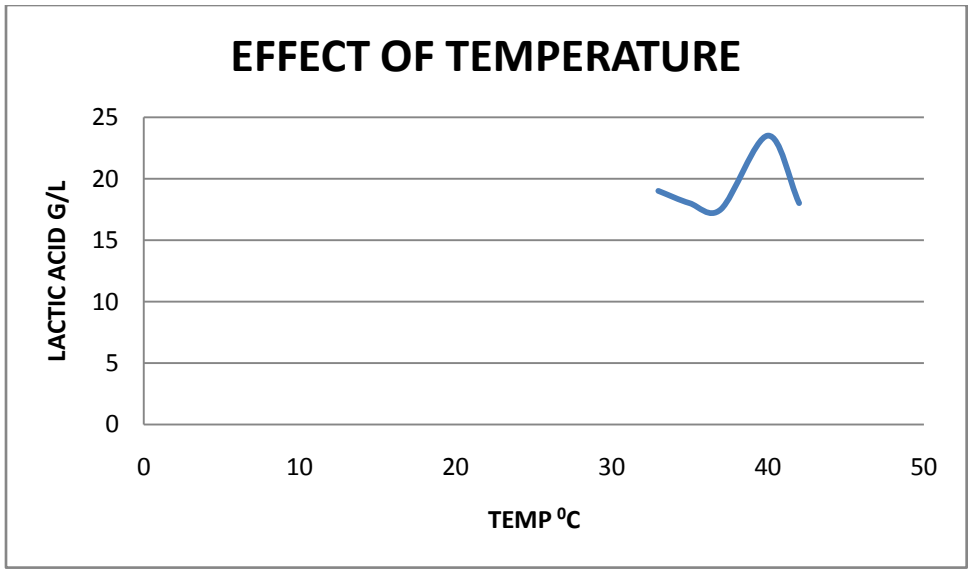

Fig.3:- Effect of Temperature on lactic acid production.

The effect of temperature on lactic acid accumulation is studied. The lactic acid accumulation is about 23 g/l at about 42^0C.

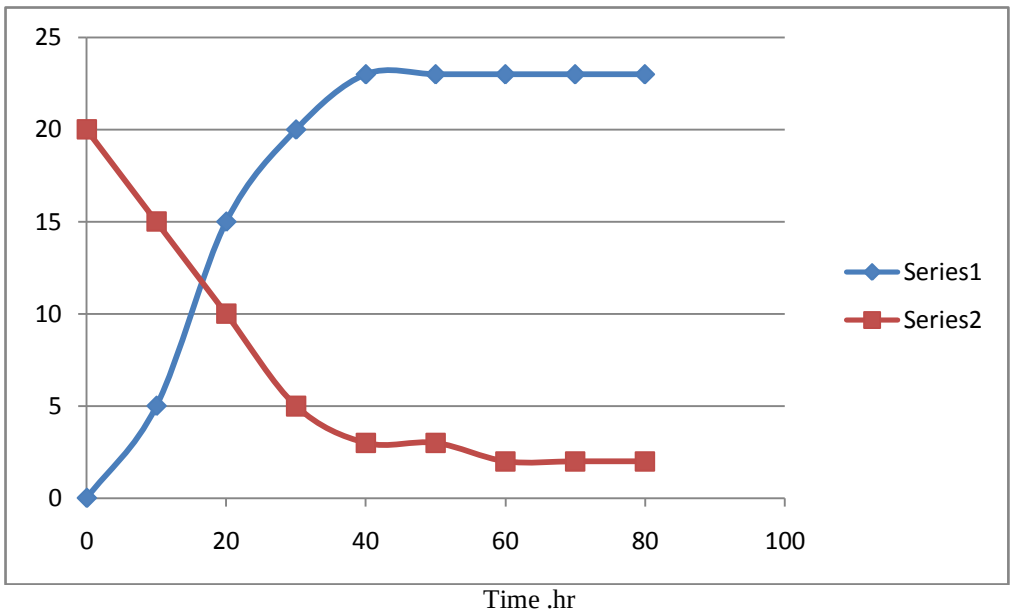

Fig.4:- Lactic acid production and total sugar depletion at 42C.series 1= lactic acid g/l and serie s2=total sugar.g/l.

The effect of temperature on the production of lactic acid from 20 g/l total sugar is examined. At 42 C 23 g/l .

The expriments are repeated till the reproduciable results are obtained. Further studies to enhance the accumulation of lactic acid .

Since mutant needs different type of medium components specially nitrogen , it needs further optimization of medium components. Therefore, eight medium components are optimized at three different concentrations by Taguchi DOE methodology. adh- mutant are more efficient than wild type since it require low amount of yeast extract and ethanol byproduct was nil as a results in 20 % increase in lactic acid with same amount of cane molasses in compared with wild type. In presence of CSL, and vitamin, yeast extract can be replaced to insignificant level without affecting the overall productivity. The addition of vitamin B12 and MnCl2, arginine and CSL increases the

lactic acid production and interact directly with the molasses. Vitamin solution 4 v/v shows enhanced in lactic acid while yeast extract and CSL shows enhanced lactic acid production at concentration 0.2 % (w/v) and 10 mL/L respectively. Yeast extract can be minimized in presence of CSL up to insignificant level (0.2 %) and but if vitamin B12 is added externally. The lactic acid production is about 23 g/l in these studies. The low accumulation of lactic acid may be attributed to lignocellulosic material present in ground nut shells.

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