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Immobilization of *Saccharomyces Cerevisiae* on Novel Matrix for Ethanol Production

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ABSTRACT

Immobilizationof yeast for ethanol fermentationhas recently gained importanceforincrease inefficiency,economic advantages and yeast stabilityoverfree cell system.Three matrices namely wood chips, eggshell membraneandhumannails were evaluated asmatrices forimmobilizationof yeast strain(NCIM 3455) and ethanol production.This paperdeals withcell immobilizationtechnique coupled with repeated batch fermentationto increasealcoholic fermentation usingyeast cells inbatch mode ina conical flask.Fermentation rates and yeast stabilityhas beenobservedto increase significantlyafterimmobilization.Forimmobilizationof yeast on humannails and eggshell membrane gluteraldehydeis used as acouplingagent and forwood chips, yeast is immobilized byadsorption. Yield of ethanol produced by immobilized cells was observed.Estimationof alcohol productionby immobilizationof yeast was done on realsubstrate,molasses. Finally,comparisonforethanol productionbyvarious immobilizationtechniques is discussed.

KEYWORDS:

Saccharomyces cerevisiae; immobilization; gluteraldehyde; fermentation; ethanol; adsorption

INTRODUCTION

Ethanol is used as an industrial solvent, as a fuel, as a alcoholic beverage since long. Use of ethanol as fuel blend is increasing exponentially over the past few years due to its high calorific value. It can either be prepared by chemical synthesis from acetaldehyde or ethylene or by the process of fermentation. Fermentation involves conversion of sugars into ethanol and carbon dioxide. Sugars are converted into ethanol and carbon dioxide by yeast cells [1]. Molasses is the residue generated after extracting edible sugar from sugarcane juice also known as blackstrap molasses. In present work molasses is been used for alcoholic fermentation using *Saccharomyces cerevisiae*strains.

Immobilization of yeast has been widely applied to industrial alcohol fermentation due to its high cell density, greater volumetric productivity, tolerance to higher concentrations of substrate and products, relative easiness of downstream processing, etc. [2]. Different matrices are currently used in laboratory and continuous study is being carried out to improve efficiency of the immobilization and ethanol production process. Very few matrices have found their way into industrial applications. Matrices currently used in industry include woodchips, sodium alginate beads, glass beads etc. Matrices such as glass or polycarbonate plates, commercial synthetic sponge, spheres of stainless steel, cotton cloth employed for immobilization of *Saccharomyces cerevisiae* by adsorption. [3, 4, 5, 6]

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Wood chips waste is generated on daily basis. Pretreatment of wood chips include size reduction using mechanical grinder, sterilization using an autoclave. Immobilization of yeast cells on wood chips is due to adsorption. Wood chips are inert towards yeast and do not hamper ethanol production. Rakin*et al*, 2009 [7], has reported that using wood chips gives thegoodefficiencyin terms of ethanol production, alcohol toleranceby yeast and stabilitytowards environmental changes.

Immobilization byuse ofsodium alginateis achemical process.Immobilization is dueto entrapment ofyeast in asemi-permeablebarrierwhich allows inflow ofnutrients and outflow of ethanol acrossthe membrane [8]. Useof sodium alginatehelps reducingdown streamingprocesscost.Immobilization ofmicrobial cells in alginatebeadswith theuse of gluteraldehydeand polyethyleniminehavebeen investigated[2, 9, 10, 11]. Immobilization ofmicrobial cells on eggshell membraneusing gluteraldehydehas also been investigated [12]. Matrices likebacterial cellulose membrane, porous cellular membranesare also beingstudied [13], [14].

This paper focuses on immobilizationofyeast cells onmatrices likewood chips, eggshellmembraneandhuman nails.It describes a comparison between different matrices for ethanol production.Immobilization techniqueslike adsorption and covalent binding havebeen tried to immobilizeyeast forethanol production. Wood chipshavebeen usedas a carrier matrixforimmobilization ofyeast cellsbyadsorptionwhile eggshellmembrane and human nails for covalent bindingofyeastcells. Comparison in terms of ethanol production for both theimmobilization method isillustrated.

MATERIALS ANDMETHODS

Microorganisms andCultureConditions:

Saccharomyces cerevisiae(NCIM no. 3455) was cultivated in MGYPmedia (NCLstandardmedia)at 29°Cfor24 hours.These cellswereinoculated in inoculum mediahavingcomposition molasses 1g,yeast extracts0.5g, $KH_2PO_4$0.5g, NH_4Cl 0.15g, $MgSO_4$0.07g, KCl0.17gin 100mldistilled water. Cells havingdifferent agesfrom 3 days upto 12 dayswerepicked upfrom theMGYPslantsand then inoculated in themedia to check maximumactivityof cells in terms ofsugar utilization. Activityof cells with varying concentration ofmolasses in themediawas also checked to studyits effecton sugar utilization. Molasses concentration was varied in the rangeof 1gm to 5 gmin 100 ml distilled water.

Growth curve analysis:

Fermentation depends on cell viability and growing state of cell, hence fermentation is done by inoculating the cells after fixed number of days, to find maximum activity of yeast cells for ethanol production. Fermentation was carried out by using cells on 3^{rd}, 6^{th}, 9^{th}, 10^{th}, 13^{th}, 14^{th}days age of inoculation. For every batch of fermentation, two flasks of 100ml media were used. The cells are inoculated from liquid inoculation media to sterilized MY media or Molasses fermentation media. Volume of inoculation is kept constant throughout the process as 5ml of inoculation per 500ml of fermentation media. Fermentation is done for 72 hours for every fermentation flask. Agitation is given as 100rpm. Sampling is done after 72 hours and centrifuged to pellet down the cells. This centrifuged liquid is used for DNS assay to find out amount of sugar reduction.

Immobilization ofCells:

Immobilizationof *Saccharomycescerevisiae*(NCIM 3455)has been studiedbyadsorptionand covalent binding.

Adsorption Method:

Wood chips weremechanicallytreated to reducethesizeto 1mm and placed in 250ml conical flask. Thesewood chips weresterilizedin an autoclave(Make:BIOTECHNICS INDIA) at 121°Cand 15psi pressurefor 15 minutes.Later, 100 mlof suspended cell mediawas introduced into the flaskin a laminarair flow(Make: ENVAIR CLEANAIR EQUIPMENT, Sr. No. 037375) and incubatedfor48 hours under continuous shaking condition at 30° C. Free Suspended cells werediscardedafter incubation time and the supports

werewashedwith glucosesolution. Duringthe washingprocess thenon- adsorbed cells were removed. Theimmobilized cells remain attachedon thematrixand later onusedfor fermentation.

Covalent Binding Method:

Eggshell membranewasgrounded and washedwith distilled water. Then, itwas dried in a hot airoven (Make: PathakElectrical Works, Sr. No. 885) at 40°Cuntil constant weight was observed. 2%,6%and 10%(v/v)gluteraldehydesolution wereprepared using citrate- phosphate buffer (pH4.7) [15]. Grounded eggshellwereintroduced in therespective gluteraldehydesolution and shaken at 100 rpm for30 minutes. Theywerethen washed with distilled waterto remove extrasolution. Thetreated eggshellmembranewas introduced in theinoculation media alongwith free cells forimmobilization.Incubation was carried out for 24 hours at 30° C under continuous shakingcondition.Free cells were removed and the matrixwas washedwith2% sugarsolution to remove anynon- immobilized cells. Thematrixwas introduced in fermentation media and theincubation was carried out for 72 hours at 30°Cunder shakingcondition at 70rpm (Make: RemiInstrumentsLtd., Cat. No. CIS.24BL). To studyreusabilityof matrix,freshsterilized mediawas again introduced into the flask containing the same matrix.

Human nails weregrounded to 1mm in dimensionand transferred toa250mlvolumetric flask. Theywerethen sterilized in a hot air oven.Increasing concentrations of gluteraldehydewas mixed with sterilized nails at30° C for30 minutes. Then, the nails were washedwith distilled waterto remove excessgluteraldehyde. Washednails were introduced into the inoculation mediain a laminarair flow(Make: ENVAIR CLEANAIR EQUIPMENT, Sr. No. 037375) and incubatedfor24 hours at 30° C undercontinuous shaking.Immobilized cells wereseparated from freesuspended cells in themediausing sterileNo.1 Wattmanfilterpaper.Immobilized cells werecarefullyrinsedwith 2% sugar

solutionto wash out the non-adsorbedcells. Fresh sterile fermentation media was then

Introducedand incubatedat 30° Cfor72 hours.Samplingwas doneand thematrixwas reusedfor next batch foralcoholic fermentation.

Sugar Estimation:

As the carbon source used in the work is molasses, the amount of sugar present in molasses before fermentation was determined. The amountof sugar leftin themedia afterutilization by yeast for ethanol production was alsoestimated byDNS assay[15].Sugar estimation was doneusing DNSassaybyusing glucoseas standard varyingin therangeof 0.33 to4 mg/ml. Standardgraphof absorbance versusglucose concentration was plotted and employed tocalculatereducingsugars in the fermentation media.

Ethanol Estimation:

To makestandardcalibration curve forethanol production reported byRaoet al, 2010 [15], was followed.In short, standard alcohol varyingin concentration of0-5mg/ml was pipetted. To this 1ml 0.1gm/ml$K_2Cr_2O_7$was added. All thetest tubes werekept in ice water and 4 mlofconcentrated sulfuricacidwasadded to each tubegentlyalongthewalls ofthe test tube. Then theoptical densitywas measured at 660 nm[15]. A sampleof 1ml was taken out from media containingimmobilized yeast cells after 48 hours. Alcoholyield wasdetermined bythe same proceduredescribed above.

RESULTS ANDDISCUSSIONS

CellGrowth:

Cells havingageof 10daysdisplayed maximum activityof sugar utilization in themedia. Hence,cells of age10 days wereused forimmobilization on the matrices.As the activity of cells was alsocheckedfor effect with varyingsugar concentration inthemedia, itwas foundthat maximum sugar utilization was in media containing3gm of molasses. Hence for further experimentation the molasses concentration in themediawas kept3gm.

Immobilization:

Immobilization ofyeast (NCIM 3455) on human nails andeggshell has been reported for the first timeusing gluteraldehydeactivation.With increasingconcentration of gluteraldehydeforyeast immobilization ethanolyieldwas found to increase. This maybe dueto theincreased strength of covalent binding.For yeast cells immobilized on Egg shell membrane, maximum ethanol production was obtained with 10% gluteraldehayde solution, whereas for yeast cells immobilized on human nails,6% glteraldehyade solution showed maximum ethanol production. Reusabilityof immobilized cells using gluteraldehydesolutiononeggshellmembraneandhuman nailshas shownethanol production with 100% efficiencyfor threecontinuousbatches usingthe sameyeast cells. Ethanol produced by yeastcells which are immobilizeon wood chips is almostconstant for repeated threebatches. Averageethanol produced is tabulated in the **Table1**. Samples were separated forthe media after fermentation and heat dried forSEM images.**Figure 1**shows SEM images ofyeast cells immobilized on wood chips byadsorption.Images also displayself aggregation ofyeast cells.

Sugar Estimation:

Total reducingsugars is averyimportant parameter in Feedstock analysisas alcoholyield is dependent on sugarcontent. This parameter is also useful indetermining process efficiencyand overallperformanceof theprocess.From commercial pointof view pricing and gradation ofFeedstock especiallymolasses isdoneon the basisofTotal Sugar Content in it.Total residual sugar concentration in real test sample, molasses, was estimated and foundto be46.31%.

Cells with ageof 10 days displayedmaximumsugar utilization and hence, these cells with ageof 10dayswereusedin thefermentation process.

Alcohol Estimation:

Alcohol producedwasquantitativelyestimatedusingpotassiumdichromatemethod. Regression coefficient forthe standardgraph was0.9931 which showsthegraph is linear in thegiven concentration range. Ethanol produced byimmobilizedyeast was calculated from $K_2Cr_2O_7$standard graph byextrapolatingtheabsorbancevalues on it.**(Table 1)**

Fermentation:

Equal volumes of fermentation mediumhavemolasses as carbonsourcewereplaced in two 250 mlflasks, which werethen inoculated with immobilized cells. Samplingfrom each flask was doneafter every48 h in order to assess theyieldof alcohol fermentation by immobilized based onyield of alcohol, amountofreducingsugar. Thepositive effect of yeast immobilization wasnoticeable with theyieldof alcohol produced byimmobilized cells was higher than that usingfreecellsuspension.Theimmobilized cellsexhibited a high sugar utilization rate.

ReusabilityofImmobilized Cells:

Immobilized cells wereused forthree continuousbatches. After incubationof cells in a batch for48 hours, themediawas taken out as sample and replaced with fresh new batch. Reusingofimmobilized cellson eggshell membrane and human nails bycovalent binding and on wood chips byadsorptionhas shown ethanol production with 100% efficiencyfor three continuous batches**(Graph 1).**Immobilization helps toreducedownstream processingcostandalso to reuse thesameviablecells which areotherwisedrained out if used in a free state.

CONCLUSIONS

Thework investigatesand provesthe feasibilityofusing eggshell membraneas a new support carrier foryeastcells. Particularly, betterfermentation results byimmobilized yeast cells than freecellsimplied thatusingeggshellmembrane, woodchips and human nails as the support carrier would havelittle effecton cellviabilityand proliferation. Therefore, the matricesdeveloped in this studycould be promising, environmentally friendlycandidate forapplication in the ethanol production industry.As thematrices used

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forimmobilization werewaste, the cost involved in upstream processinginreducedas compared to synthesizedmatrices likebacterial cellulose membrane.

TABLE

Table 1: Average ethanol produced from immobilized cells for three repeated batches

Sr. No.	Substrate	Batch	Sugar remained (mg/L)	Ethanol yield (g/L)
	Woodchips	1	0.776	8.75
		2	1.13	7.58
		3	1.54	6.36
	Human nails 2% gluteraldehyde	1	1.40	6.89
		2	0.83	8.33
		3	1.64	6.24
	Human nails 6% gluteraldehyde	1	0.96	8.25
		2	0.677	9.285
		3	1.29	7.13
	Human nails 10% gluteraldehyde	1	1.19	7.25
		2	1.12	7.33
		3	1.38	6.89
	Eggshell membrane 2% gluteraldehyde	1	1.22	7.21
		2	0.77	8.75
		3	0.41	9.97
	Eggshell membrane 6% gluteraldehyde	1	1.19	7.25
		2	0.903	8.25
		3	0.096	10.99
	Eggshell membrane 10% gluteraldehyde	1	0.903	8.25
		2	0.322	10.24
		3	0.096	10.99

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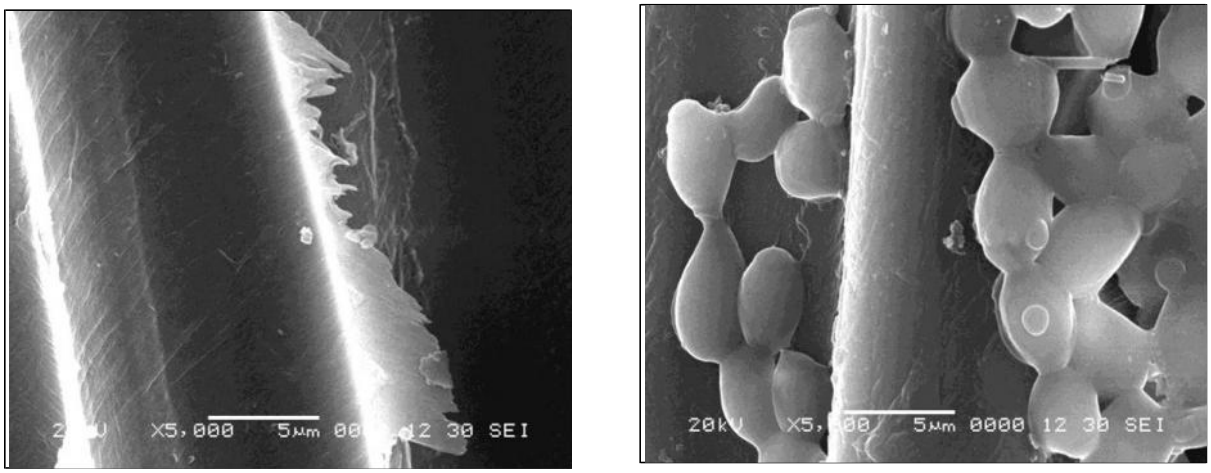
FIGURES

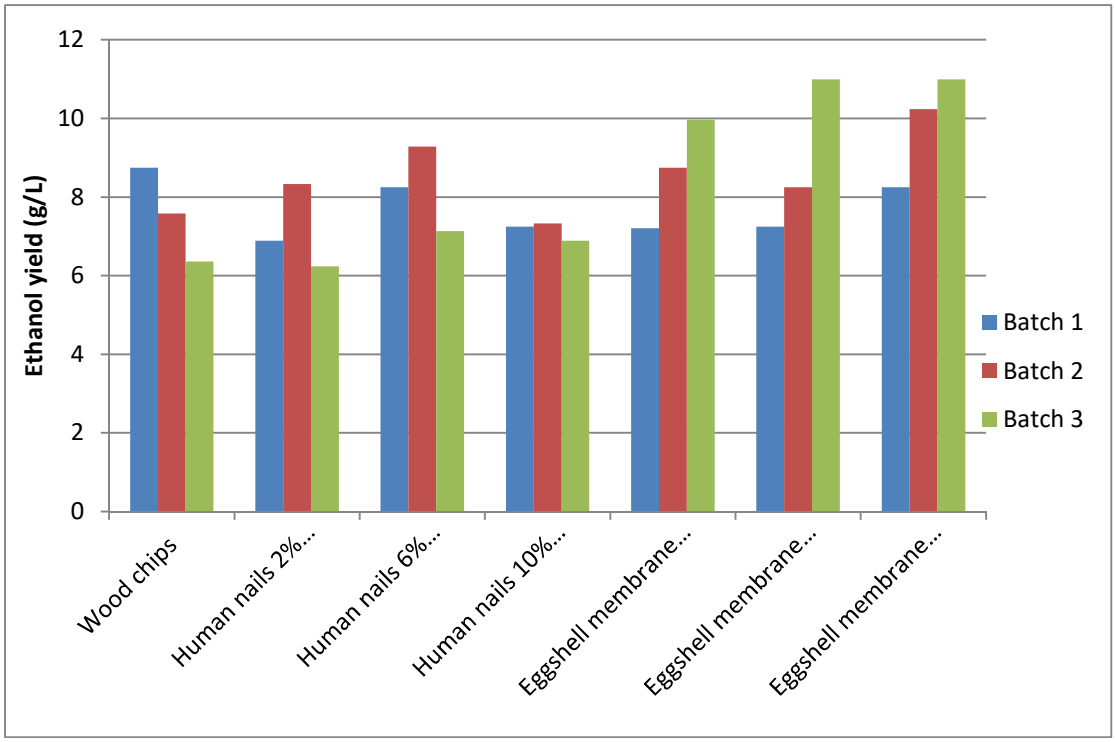


(a) (b)

Figure 1: SEM images illustrating (a) wood chips before immobilization of yeast cells. Images (b) illustrate the morphology of yeast cells adsorbed on wood chip

GRAPHS

Graph 1: Ethanol yield from immobilized cells for three repeated batches with different matrices



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