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CONSTRUCTION OF XYLOSE ASSIMILATING YEAST HYBRIDS THROUGH GENOME SHUFFLING

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ABSTRACT

Production of bioethanol from lignocellulosic biomass is gaining much research interest because of its abundant supply. *Saccharomyces cerevisiae* is The commonly used industrial yeast for ethanol production but it lacks the property to ferment pentose sugars mainly xylose present in lignocellulosic materials. In this study, the hybrid yeast strains are developed by genome shuffling process between the xylose sugar fermenting yeast *Pichia stipitis* and hexose sugar fermenting yeast *Saccharomyces cerevisiae*. Lyticase enzyme is used to establish viable protoplasts from both the parental yeast strains. Factors affecting physic chemical properties *viz.,* protoplast isolation, enzyme concentration and incubation time were investigated. The optimal parameter for the protoplast release of *S. cerevisiae* and *P. stipitis* include 700 µg/µl Lyticase for 60 min and 700 µg/µL Lyticase for 120 min respectively. The maximum protoplast formation ratios were 98.75% and 82.24% for *S. cerevisiae* and *P. stipitis* respectively. The frequency of the protoplast fused hybrids was carried out by using Polyethylene Glycol (PEG) as fusogen. Fused hybrids were produced by use of 35% (w/v) PEG 4000, optimized conditions of protoplasts fusion of *S.cerevisiae* and *P. stipitis* genome shuffling was achieved at a rate of 80.90% with fusion of 20 min. Further, these hybrids produced through genome shuffling will be further evaluated for production of bioethanol using lignocellulosic materials.

KEYWORDS: **Bioethanol, Lignocellulose, *Pichia stipitis*, Polyethylene glycol, Protoplast, *Saccharomyces cerevisiae***

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INTRODUCTION

With the inexorable exhaustion of the world's fossil resources, there has been an increasing interest worldwide in search for alternative sources of energy[1]. Renewable energy is sustainable and its sources include wind, solar, biomass, geothermal and hydro, all of which occur naturally and are inexhaustible unlike fossil fuels. Of this the use of biomass energy has the potential to greatly reduce greenhouse gas emissions. Biomass or bioenergy the energy from plants and plant-derived materials, which include food crops, grassy and woody plants, residues from agriculture or forestry,oil-rich algae and the organic component of municipal and industrial wastes. Ethanol production from the above bioenergy material through the fermentation process has a pivotal role in the last few years due to its increased use as fuel and as a complement to gasoline[2]. The production of biofuels is gaining much research interest as it is less toxic, readily biodegradable and produces less air pollution compared to fossil fuels[3] .Commercially bioethanol is produced from cellulosic and lignocellulosic materials, due to its readily availability,and ability to produce cleaner fuel[4]. But the production of bioethanol from biomass is challenging as the biomass is composed of cellulose, hemicellulose and lignin polymers. The cellulose and hemicellulose constitutes the main source of sugars like 6 carbon sugar glucose (6C) and five carbon sugar xylose (5C) that can be fermented to ethanol by use of microbes like yeast and bacteria. But the major challenge of the microbes in the bioethanol production is to coferment both glucose and xylose present in lignocellulosic biomass. The ability of microorganisms to ferment xylose to ethanol has been investigated by some researchers[5] since *Saccharomyces cerevisiae*, which is the common industrial microorganism for ethanol production, cannot ferment pentose sugars[6]. *Pichia stipitis* is one of the natural xylose-fermenting yeast strains and an useful species for direct fermentation of xylose to ethanol [7] But *Pichia stipitis* has low ethanol and sugar tolerance[8] . The efficient fermentation of xylose leads to economically viable processes for production of biofuels such as ethanol from various lignocellulosic materials [9]. To develop hybrid yeast strains various techniques like protoplast fusion[10], recombinant DNA technology[11], random and site directed mutagenisis [12,13] were employed. Among these process Protoplast fusion is one of the common method as it is simple and also offers an advantage of exchanging the genetic material simultaneously at different positions throughout the genome without the necessity of the genomic information of the species [14]. Protoplast fusion plays a significant role in genetic engineering because it resolves the limitations of genetic exchange imposed by conventional mating systems and also allows the exchange of relativelylarge segments of genomic DNA[15]. Protoplast fusion is a modest and extensively used process to form the improved varieties of industrial yeast strains.This technique has successfully resulted in improving the acid tolerance in *Lactobacillus*[16-18], production of hydroxycitric acid in *Streptomyces*[19], stable and good performing fusants are formed which can utilize the maximum amount of reducing sugars in the media[20]. In this present work we assessed physico- chemical parameters requisite to carry out protoplast isolation and fusion from the parental strains of *Saccharomyces cereviseae* and *Pichia stipitis*. Finally the optimum conditions are established in production of yeast hybrids *via* genome shuffling process towards effective utilization of hexose and pentose sugars available in biomass.

MATERIALS AND METHODS

Yeast parental strain: *Saccharomyces cerevisiae* (NCIM-3090) and *Pichia stipitis* (NCIM-3497) were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India.

Culture Media

Parental strains of Yeast strains are cultured on YPD Yeast Peptone Dextrose medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1}dextrose and 20 g L^{-1} agar), whereas for regenerating the hybrids regeneration medium *i.e.,* YPD medium supplemented with 0.6 M KCl and 0.67 % Yeast nitrogen base were used, whereas for regeneration of the fusants regeneration overlay media consisting of regeneration medium with agar content reduced to 10 g L^{-1}) was used in this study.

Preparation of protoplast

The method described by Curran and Bugeja (1996) was adopted to isolate the protoplasts from *S.cereviseae* and *P.stipitis* with slight modifications[21]. The two parent yeast cells were separately grown in 25 mL of YPD medium at 30^0Ċ until they reached the log phase. 10 mL of culture cells were harvested by centrifugation at 6000rpm for 5 mins. The pellet was washed with 0.1M Potassium phosphate buffer at pH 7.5 and harvested again. The pellet was suspended in lysis buffer containing lysing enzyme i.e.,lyticase (Sigma Chemicals, St. Louis, USA) and 50µl of β- mercapto-ethanol and then incubated at room temperature. The cell wall lysis and protoplast release was monitored for every 30 mins and observed using Inverted microscope (Optika Model XDS-1r). The protoplast number was counted using a haemocytometer. The protoplast suspension was then centrifuged at 500 rpm for 15 mins and protoplasts were collected and suspended in osmotic buffer solution (0.6 M of sorbitol in 0.1M phosphate buffer). All the above experiments were carried out in triplicates. Protoplast yield was calculated by using the following equation

$$\text{Protoplast yield} = \frac{\text{Number of protoplast released}}{\text{Number of cells incubated with lysing enzyme}} \text{ X } 100$$

The effect of physico -chemical parameters *viz.,* concentration of lysing enzyme, Lysis time and effect of osmotic stabilizers which affect the protoplast yield from yeast parental strains were investigated. The optimum conditions that are required for viable protoplast formation were recorded.

Protoplast fusion and regeneration

The protoplasts of *S. cerevisiae* and *P. stipitis* were mixed in equal volumes and centrifuged at 500 rpm for 5 min in an osmotic buffer solution. The pellets of each strain were suspended in fusion buffer solution (33 % PEG (MW 4000) in STC buffer (0.6 M sorbitol; 10 mMTris–HCl; 10 mM $CaCl_2$; pH 6.5). To establish the favourable conditions for the fusion of protoplasts, pellets are subjected to various concentrations of PEG (4000) and at different pH. The resultant suspension was incubated at 30^0C at 100 rpm. Every 10 min 0.25 mL of the suspension was withdrawn. The fusion of the protoplast was supervised under an inverted microscope. The fused protoplast numbers were counted under haemocytometer. The fusion frequency was determined by the following equation,

$$\text{Fusion frequency} = \frac{\text{Number of protoplast fused}}{\text{Total number of protoplasts}} \text{ X } 100$$

Regeneration of the fusants

Fused protoplast suspension of 0.5 mL was mixed with 10 mL of molten regeneration overlay medium and poured as a thin upper cover on the regeneration medium plate. The plates were incubated at 27^0 Ċ for 3 - 4 days. The observed colonies were streaked on selective media consisting of Yeast extract, Peptone, Xylose (YPX) and Yeast extract, Peptone, Dextrose (YPD) plates to verify their hybrid nature. Further, these successful hybrids were tested for better utilization of hexose and pentose sugars in the fermentation broth for the production of bioethanol.

STATISTICAL ANALYSIS OF DATA

The experiments *viz.,* lysing enzyme concentration , lysis time osmotic stabilizers, PEG concentration, PEG exposure time were done in triplicate t-Test analysis was performed and the results were shown at mean ±SD of atleast three independent experiments .Significance set as $p < 0.05$.

RESULTS AND DISCUSSION

Genome shuffling is a powerful tool to construct hybrid strains with desirable phenotypes. In this approach the genome simultaneously changes at different positions without the necessity to have the prior knowledge of the actual genome sequence. Lignocellulosic biomass is convertible to bioethanol by fermenting the sugars present in it[22]. The biomass mainly consists of cellulose, hemicellulose and lignin polymers. The cellulose and hemicellulose constitutes the main source of sugars like 6 carbons sugar glucose (6C) and five carbon sugar xylose (5C). In this study through genome shuffling we produced hybrids from parental yeast strains that are capable of utilizing both hexose and pentose sugars present in the fermented broth. These hybrids are further tested on lignocellulosic biomass to evaluate its ethanol production. The physico chemical conditions play a pivotal role in the genome shuffling process. Hence various parameters *viz.,* concentration of enzyme, lysis time and other factors were studied to establish the optimum conditions for protoplast formation which resulted in the hybrid strains that can convert both hexose and pentose sugars to ethanol.

Protoplast isolation

The two parental strains of *S.cereviseae, P.stipitis* were treated separately with enzyme lyticase 10KU for protoplast isolation. Both the parental yeast strains are oval shaped and the protoplasts formed are spherical, So freshly formed protoplasts can be easily differentiated from others. Hemocytometer was used to count the number of protoplasts formed

Effect of lysing enzyme concentration

The lysing enzyme concentration influences the cell wall lysis and also in the formation of the viable protoplasts. Hence, various concentrations of lytic enzyme were used to determine the effect of lysing enzyme on two parent strains of yeast cells in the range of 100 – 800 µg/µl. As indicated from the figure 1A, the number of protoplasts formed from *S. cerevisiae* was found to increase gradually until 500 µg/µl, it almost remained constant until 700 µg/µl and declined with further rise in the concentration. Similar fashion was also followed for *P.stipitis* other parent strain in this study and the maximum yield was achieved at 700 µg/µl (Fig 1B).Different concentrations of the Lytic enzyme effected the protoplast yield in both the yeast cells, Similar observations were also made by Balasubramanian et al[23] while isolating protoplats of *Trichoderma harzianum* and *Trichoderma viride* strains. However, increased concentration of enzyme was associated with the decline in the protoplast yield. This is due to the toxic effect of the enzyme which resulted in lysis of the protoplast.Excess enzyme hasno further effect on the left over cell wall due to limited site access. Lower concentrations resulted in fewer number of protoplast where as higher concentration of enzyme resulted in bursting of the protoplast and further cells disintegrated immediately[24]. In our study lyticase at a concentration of 700 µg/µl resulted in optimum yield of protoplasts i.e., 98.75% and 82.24% in *S.cerevisiae* and *P. stipitis* respectively [Fig 2].

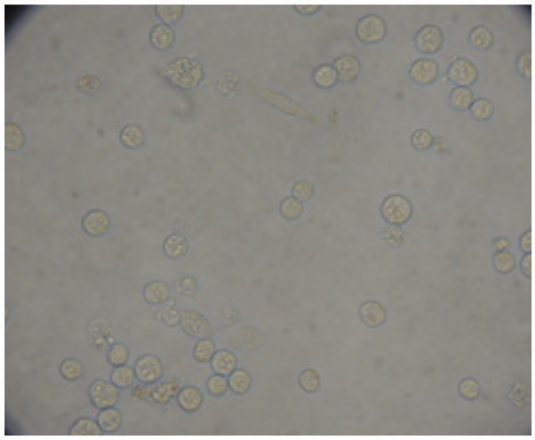

Figure 1A
Isolated protoplasts from S.c

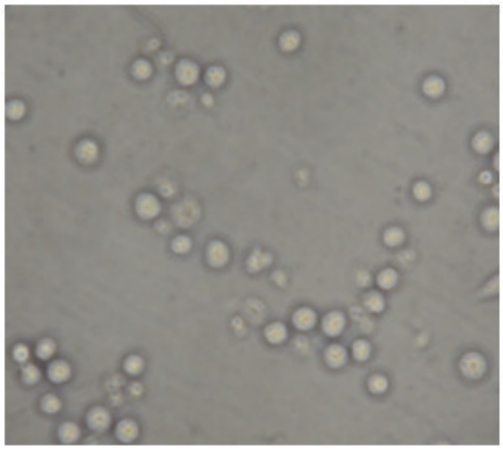

Figure 1B
Isolated protoplasts from P.s

Figure 1
Protoplast of S.cereviseae and P.stipitis formed when treated with lyticase enzyme

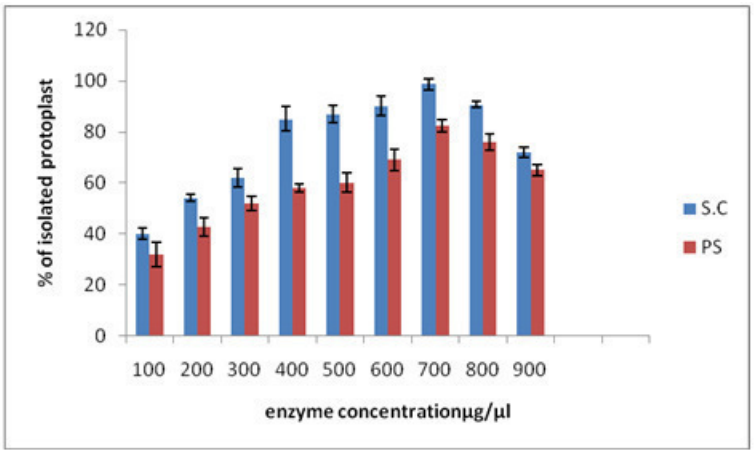

Figure 2
Effect of enzyme concentration The cumulitative Data of each assay was done from three independent experiments and shown has mean± SEM (n=3) , p<0.05

Effect of lysis time

One of the most important element which plays a pivotal role in protoplast yield is the time of exposure of lytic enzyme to the yeast cells. Bacon *et al* studies revealed that continuous enzymatic incubation of the cells resulted in the impairment of the nascent protoplasts of yeast cells[25]. To study the optimum incubation time both the parent strains of yeast cells were incubated at various time intervals of 30-210 min with 700 µg/µl lysing enzyme lyticase to examine the maximum protoplast yield. The release of viable protoplasts and the amount of protoplasts were observed in microscope and hemocytometer respectively. The maximum protoplast yield for *S.cereviseae* (96.4%) was obtained at 60 min whereas for *P.stipitis* (84.25%) maximum yield was achieved at 120 min. Continuous exposure to lysis enzyme leads to shrinkage of the protoplasts which was observed after 210 mins in both the cases [Fig 3]. Henceforth, the optimum lysis time for *S.cereviseae* and *P. stipitis* has been recorded as 60min and 120 min respectively without any traces of cell wall on cells. Darling *et al*., 1969 show that the alteration in the sensitivity of different yeast strains were governed by the enzyme degradation, along with the presence of older cell walls which are resistant to enzymatic degradation[26]. Kumari 2012 studies exhibits the difference in the composition and thickness of the cell wall attributes to the formation of the protoplasts in yeast strains[27]

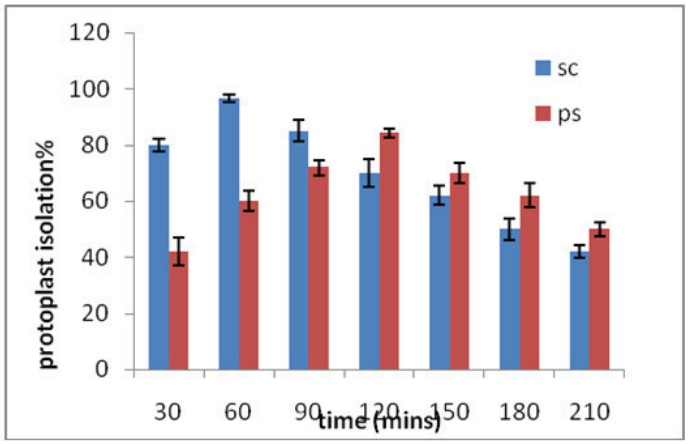

Figure 3
Effect of time on protoplast isolation: The cumulitative data of each assay was done from three independent experiments and shown has mean± SEM (n=3) , p<0.05

Effect of Osmotic stabilizers

To avoid the lysis of the protoplasts and to enhance the stability of the protoplasts addition of osmotic stabilizers is very important[28]. Different osmotic stabilizers were tested and found that $MgSO_4$, mannitol and sorbitol are the most suitable stabilizers[29] for isolation and fusion of protoplasts of yeast cells.Due to enzymatic degradationof the cell walls, the complete cell content is sealed only by the cell membrane. Further, survival of the protoplasts is achieved only when they are preserved in a hypertonic medium which provide them the osmotic stability. In this investigation, osmotic stabilizers such as 0.6M KCl, 0.6 $MgSO_4$ and 1.2 M sorbitol were used to investigate their effects on protoplast yield of both the parental strains. The maximum protoplast yield was observedby the use of Sorbitol at a concentration of 1.2M and achieved 96.6 and 86.9% protoplast yield in *S. cerevisiae* and *P.stipitis* respectively [Fig 4]. Similar reports were also made by Ezeronye and Okerentugba (2001)while isolation of protoplasts in *Saccharomyces cerevisiae* and *Candida tropicalis*[30]. Viability of the protoplasts is decreased when the osmotic stabilizers if altered or optimal concentrations were not maintained. Further, irregularity in the cell membranes and decreased number of protoplasts and this may be due to the imbalance of osmotic pressure around the protoplasts.

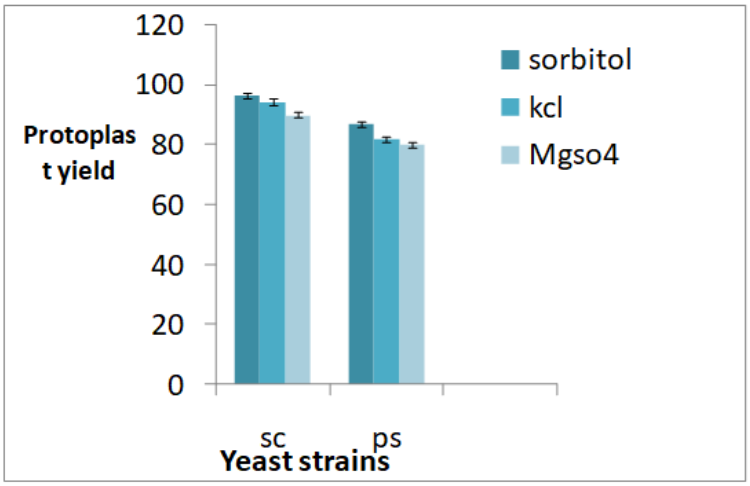

Figure 4
Effect of osmotic stabilizers: The cumulitative data of each assay was done from three independent experiments and shown has mean± SEM (n=3) , p<0.05

Protoplast fusion

Effect of peg concentration

Due to the presence of similar charges on the surface of the protoplast (-10mV to -30mV) they tend to repel each other. Fusogens are vital for the fusion of the protoplasts as they reduce the electronegativity and leads to fusion of protoplast. One of the most efficient fusogen mediated protoplast fusion is by using chemical Polyethylene glycol (PEG) which was discovered by Kao and Michayluk from Canada[31]. PEG is toxic to cells at higher concentrations greater than 50% and exposure

for longer period of incubation[32], where as lower concentrations decrease the yield of fused protoplasts. Thus the optimum concentration and time of exposure to PEG are important for efficient fusion of the protoplasts. To establish the optimized condition for the fusion of protoplast of *S. cerevisiae* and *P. stipitis*, PEG (Molecular weight 4000) with different ranges of concentrations (25-45%) were explored in our study. Use of lower concentration of PEG 4000 (<30%) resulted in swelling and ultimately burst of protoplasts[Fig 5c]. In contrast the higher concentration (>35%) of PEG4000 had adversative effect on protoplasts resulting in aggregation and over clumping of protoplasts. Moreover shrinkage of protoplasts was also observed with the increased concentration[23]. In our study we observed maximum fusion of protoplasts is at 35% PEG concentration in both the yeast parental strains [Fig 6].

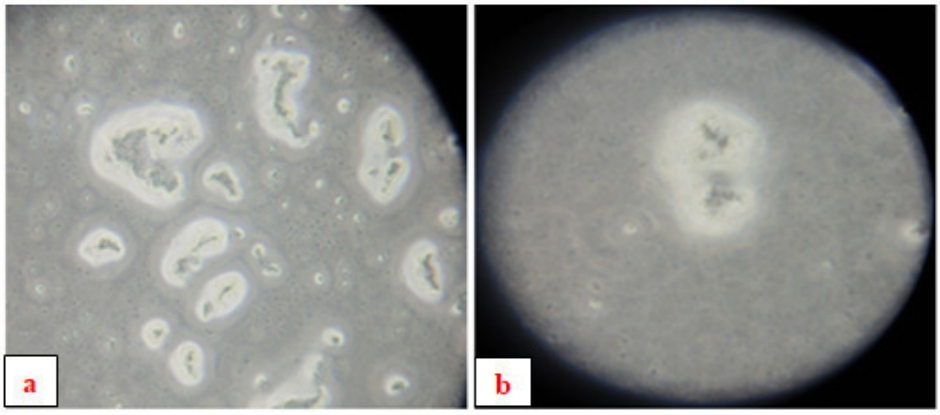

Figure 5 a, b
Fusion of the Protoplasts

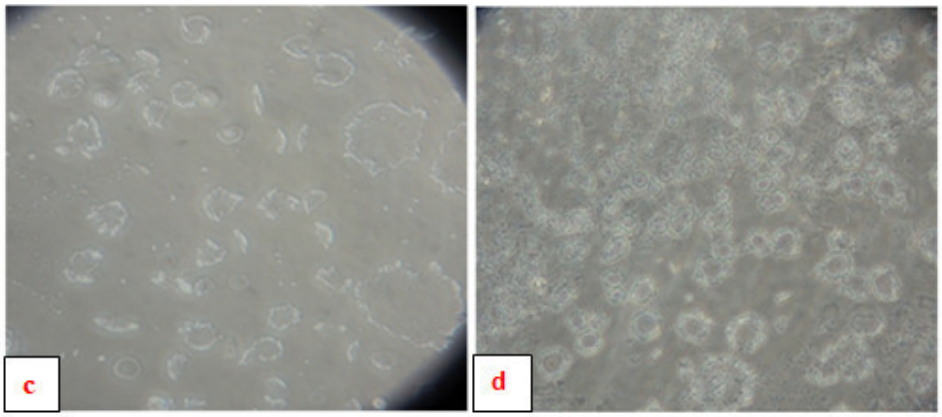

Figure 5 c, d
Burst of Protoplasts

Figure 5
Fusion and rupture of protoplasts in presence of Polyethyleneglycol, In figure a, and b depicts the fusion of the two parental strains of S.cereviseae and P.stipitis in the presence of 35% PEG4000. Similarly when the protoplasts are exposed to lower concentration of PEG4000 burst of protoplasts occurred (Fig c , d) .

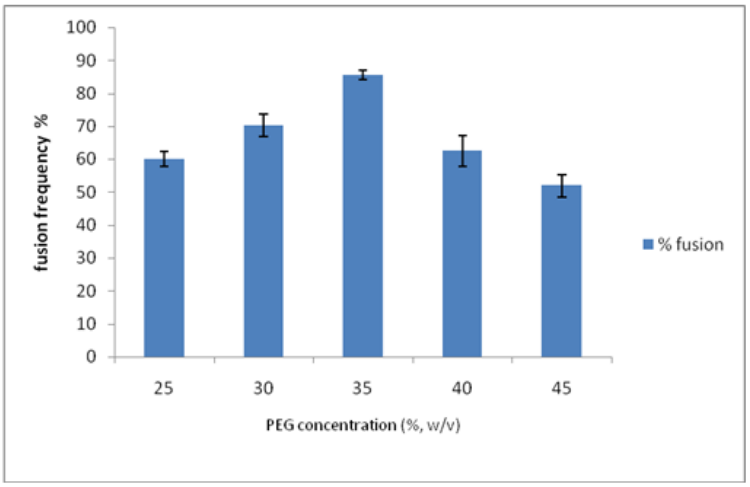

Figure 6
Effect of PEG on protoplast fusion: The cumulitative data of each assay was done from three independent experiments and shown has mean± SEM (n=3) , p<0.05

Effect of PEG contact time

The exposure time of protoplasts to PEG 4000 has a significant influence in the formation of fusants of yeast cells. The prolonged incubation with PEG resulted in toxic to the yeast cells[33]. The optimum exposure of the cells to PEG helps in enhancing the transformation efficiency and frequency of cells by increasing the permeability of membrane[34]. In this study the fusion time was selected from 10min to 40 min. The outcome of the experiment proved that the maximum fusion occurred when protoplast are treated with PEG 4000 for 20 mins. When they are allowed to expose for less than 20 mins there was adequate time for all the protoplasts to fuse. When they are exposed for more than 20 mins dehydration of the protoplasts occurred which ultimately results in burst of protoplasts[35]. At 20 minutes of incubation in PEG the highest frequency for fusion between *S.cereviseae* and *P.stipitis* is recorded as 80.9%.Boni et al., reported , if the protoplasts are exposed for 40 mins there is a decline in the fusion frequency which leads to shrinkage of the protoplasts[36].Similar observation was also reported by Kao et al. stating the decrease in protoplast fusion frequency and formation of viable colonies on prolonged incubation of protoplasts[37].

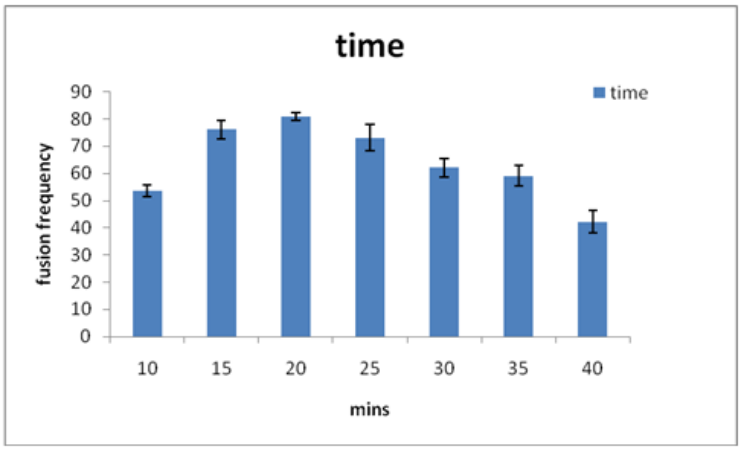

Figure 7
Effect of PEG exposure time : The cumulitative data of each assay was done from three independent experiments and shown has mean± SEM (n=3) , p<0.05

CONCLUSION

The main aim of our study is the production of hybrid yeast strains *via* genome shuffling to produce bioethanol from lignocellulosic biomass which consists of pentose and hexose sugars. Utilization of all the sugars present in biomass by efficient microbe or hybrid strain during the fermentation process is useful and economical for the production of biofuels. Different hybrid yeast strains were produced by the fusion of protoplasts obtained from *S. cerevisiae* and *P.stipitis* using Lyticase enzyme. Optimum parameters *viz.,* enzyme concentration, time of exposure and PEG

concentration were studied for the formation of hybrids which are capable of fermenting pentose and hexose sugars at the same time.

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CONFLICT OF INTEREST

Conflict of interest declared none

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