

Full Length Research Paper

Intergeneric protoplast fusion of yeast for high ethanol production from cheese industry waste – Whey

Krishnamoorthy, R. Narayanan^{*}, K. Vijila and K. Kumutha

Department of Agricultural Microbiology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India, Pin: 641 003.

Accepted 3 March, 2010

In this study, Intergeneric protoplast fusion of the yeast cultures *Viz.*, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* was carried out for enhancing production to ethanol temperature tolerance and lactose utilizing characters in a single stain. *S. cerevisiae* (Parent 1) has ethanol tolerance, whereas, *K. marxianus* (Parent 2) is temperature tolerant and also has lactose utilizing capacity. Twelve fused cultures were obtained by protoplast fusion. Fused cultures recorded higher DNA content than the parent strains, which showed complementary banding pattern of two parental Strains. SDS-PAGE confirms the presence of HSP 70 in the fused culture, which is responsible for temperature tolerance. Fermentation of cheese whey was carried out with two parental and fused cultures. The results revealed that the ethanol production was higher with fused culture (12.5%, with 18.09 g/l of biomass) after 72 h of fermentation. Parent 1 showed poor growth on the cheese whey medium, but growth of the Parent 2 was inhibited when the ethanol production reached 6%.

Key words: Marker selection, protoplast fusion, RAPD, SDS-PAGE, whey.

INTRODUCTION

Natural energy resources such as petroleum and coal have been consumed at high rate over the last decades. The heavy reliance of the modern economy on these fuels is bound to end, due to their environmental impact and to the fact that they might eventually run out. Therefore, alternative resources such as ethanol are becoming more important. Bio-ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of the fossil fuels.

Some biological processes have rendered possible route to produce ethanol in large volume using the cheap resources (Gunasekaran and Raj, 1999). The massive need of fuel ethanol in the world requires that, its production technology should be cost-effective and environmentally sustainable. For current technologies employed at commercial level, the main share in the cost structure corresponds to the feedstocks (above 60%)

followed by the cooling cost of the fermentor (Sing and Lindquist., 1998). High energy input is required to maintain temperature between 25 and 35 C in fermentation process to maximize ethanol production and prevent irreversible heat inactivation of yeast cells.

Many opportunities may be explored using different cheaper renewable waste materials with a lot of usable substrate (for example, whey, agricultural food waste, wood chips, molasses and newspaper waste) for microorganisms to grow upon and then produce useful products for society.

The production of cheese has reached 11 - 12 x 10⁶ tones per year and liquid whey produced in these processes has reached around 10⁸ tones per year. This whey contains 5 - 6% lactose, 0.8 - 1% protein and 0.06% fat (Kosikowsk, 1979). However, disposal of whey without expensive sewage treatment can represent a source of water pollution because it's high BOD which is 50,000 ppm. This is one of the least expensive Carbon source for ethanol production.

Although, earlier attempts were made to use lactose for alcohol production, the major problem was the inability of *Saccharomyces cerevisiae* to ferment lactose. *Kluyveromyces marxianus* is known to ferment lactose (Ferrari et al., 1994).

^{*}Corresponding author. E-mail: krnarayanan_2001@Yahoo.com.
Tel: 0422 6611294. Fax: 0422 6611462.

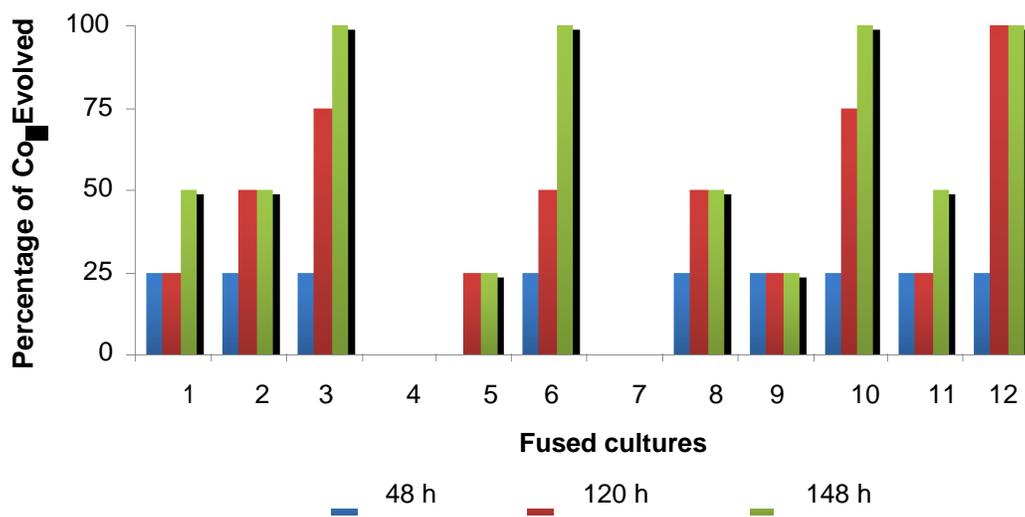


Figure 1. Percentage of CO₂ production by the recombinant yeast strains at 42 C.
*1-12 - Fused culture 1 to 12

However, it has been shown that only a fraction of the available lactose is converted to ethanol, possibly due to ethanol inhibition. *S. cerevisiae* cells can grow in high ethanol concentration, but it does not have capacity to utilize lactose as carbon source and high temperature tolerance like *K. marxianus*. To overcome the above-mentioned problems, a genetically engineered strain of *S. cerevisiae* that expresses -galac -tosidase activity was reconstructed with the capabilities for the bioconversion of lactose in whey into ethanol and temperature tolerance. Successful attempts in this respect yielded the recombinant yeast strains that were developed by intergeneric protoplast fusion of *S. cerevisiae* and *K. marxianus* (Figure 1).

METHODOLOGY

Collection of standard cultures and genotypic difference

S. cerevisiae (MTCC 181) and *K. marxianus* (MTCC 188) were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. Five differentiating characters viz, antibiotic tolerance (Barnett et al., 1986), crystal violet tolerance (Lin et al., 1985), ethanol tolerance, temperature tolerance (Banat et al., 1992) and carbon source preference (Stvitree et al., 2000) were used to differentiate two parental cultures.

Protoplast formation and fusion (Farahnak et al., 1986)

The two parental cultures were inoculated in 100 ml YEPD (Yeast extract- 10.0 g, Peptone – 20 g, Glucose - 20.0 g, Distill water – 1000 ml) broth and incubated at 35 C for 24 h. Five ml of the 24 h old parent cultures (~ 4 x 10⁷ cfu per ml) were taken (three set) in centrifuge tube and centrifuged at 6000 rpm for 5 min. The

supernatant was discarded and pellet was resuspended in 1 ml of protoplasting solution with different concentration (2, 5 and 10 ppm) of cell wall lysing enzyme (Glucanex) (Petit et al., 1994). This suspension was kept at 35 C for 1 h with occasional shaking. After 1 h the protoplasts were harvested by centrifugation (6000 rpm for 10 min), washed three times with protoplast buffer and resuspended in 1 ml of same buffer.

Protoplasts of each parental culture suspended in 5 ml of protoplast buffer was taken and added into Eppendorf tube containing 1 ml of PEG solution. This mixture was incubated in room temperature for 20 min, followed by centrifugation at 6000 rpm for 5 min and pellet was resuspended on 1 ml of protoplast buffer. Fused protoplasts in one ml of this buffer were transferred to 10 ml of regeneration medium taken in conical flask and incubated at 30 C for 72 h.

Screening of the fused cultures

The fermentation ability of the fused cultures was assessed (Van der walt, 1970) by growing the cultures at 42 C in the test tubes containing 10 ml of YEPD medium with lactose and 10% ethanol. The percentage of CO₂ evolved was calculated by the amount of CO₂ filled in the Durham's tube after 48, 72 and 120 h of fermentation. Based on the Durham's tube, amount of CO₂ filled in the percentage was recorded as 25, 50, 75 and 100%. Genotypic stability of the recombinant cultures was tested by streaking the slant cultures on the YEPD medium with markers. This was assessed on 0, 2, 4, 6, 8, 10, 15 and 20 weeks after fusion (Dziuba and Chmielewska, 2002).

Confirmation of protoplast fusion

The genomic DNA of the parental and fused culture was extracted by the method given by Melody (1997) with slight modifications. The Nanodrop® ND- 1000 was used to quantify the DNA content and random amplified polymorphic DNA (RAPD) was carried out as per the method given by Martins et al. (1999).

Table 1. Differentiating characters of parental cultures.

Yeast cultures	Cycloheximide tolerance (ppm)	Crystal violet tolerance (ppm)	Temperature tolerance (C)	Carbon source	Ethanol tolerance (%)
Parent 1	< 10	>100	39	Glucose	>14
Parent 2	>100	<10	42	Lactose	<10

Parent 1 – *S. cerevisiae*

Parent 2 – *K. marxianus*

SDS -PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

The protein extraction was done by the method described by Saumya and Hemchick, 1983 and it was estimated by Lowry's method (Lowry et al., 1951). The extracted protein samples were used in SDS -PAGE.

Whey fermentation

Substrate preparation (Mahoney et al., 1975)

Cheese industry waste was collected from Acres wild organic cheese making farm, Coonoor, Nilgris. The whey was heated at 90 C for 15 min, cooled and centrifuged at 1000 rpm for 10 min and removed the coagulated proteins. The whey medium containing 4.8% lactose was supplemented with 0.03 g K₂HPO₄, 0.05 g yeast extract per 100 ml of whey. The medium was adjusted to pH 5.5 with 0.01 N sulphuric acid, autoclaved for 15 min at 121 C, cooled to 30 C and used for the fermentation.

Fermentation and analysis

Fermentation using the parental and fused cultures was conducted in 250 ml of Erlenmeyer flasks, which contained 100 ml of cheese whey medium (CWM) (Mahoney et al., 1975) . In the medium 1.0% of inoculum, which contains approximately 40 x 10⁶ cfu per ml, was added and incubated in shaker at 42 C, 100 rpm. Biomass, lactose content and ethanol was estimated calorimetrically.

RESULT AND DISCUSSION

In order to differentiate the hybrids from parent cultures, five different set of parameters were taken into account. The first one is that anti-fungal antibiotic resistance characters of the both parental cultures were assessed. It has been found that parent 2 has tolerance to high level of cycloheximide concentration compared to parent 1 (Table 1) . Tahoun et al. (1999) used cycloheximide, imazilil to differentiate the parental cultures *S. cerevisiae* ATTC 4129 and *K. fragilis* CBS 683. The results of the *S. cerevisiae* showed susceptibility to cycloheximide even at low concentration and *K. fragilis* was susceptible to imazilil.

As the second marker, the temperature tolerance was taken in to account. Table 1 represents a comparison of parental strains to different temperatures which indicated that, parent 2 grown well in 42 C compared to parent 1.

Dziuba and Chmielewska (2002) differentiated the parental strains *S. cerevisiae*, *Pachysolen sp* and *Candida shehatae* based on the temperature tolerance. They observed that *S. cerevisiae*, *Pachysolen sp* showed tolerance upto 42 C and *C. shehatae* was sensitive to high temperature. The ethanol tolerance capacity of the parental strains were tested in the parent study and it was found that parent 1 showed tolerance to high ethanol concentration (12%) compared to parent 2 (6%). Ethanol has long been known to inhibit the growth of yeast at high concentration. Holzberg et al. (1967) observed that a threshold concentration of ethanol (about 0.6 M) below which there was no inhibition and above that level inhibition followed a linear pattern. The above finding supported the result of the present investigation; the parent 1 was able to grow upto 12% ethanol concentration. However, the decreased growth was observed above 12% concentration.

Screening of fused culture

Twelve fused cultures were taken from fusion studies, in which the cultures FC3, FC6 and FC10 with good fermenting ability were selected and there strains took 120 h (Figure 1) to fill the Durham's tube. The fused culture FC12 was the best lactose fermenting culture, as Durham's tube was filled within 72 hr. FC1, FC2, FC5, FC8, FC9 and FC11 were found to be poor in fermenting the lactose. The key issue in the assessing the usefulness of recombined strains are the ability to consume lactose in the medium, high ethanol and high temperature tolerance. Based on the growth and CO₂ evolved, the fused culture FC12 was selected as the best culture. Similarly Dziuba and Chmielewska (2002) found that 6 out of 18 hybrids of *S. cerevisiae* CD 43 and *C. shehatae* ATTC 58779 have not shown the ability to convert the xylose to ethanol. The hybrids CD43-7 and CD 43-9 were characterized as the best converting ability, because they finished the fermentation after 120 h.

The stability was assessed on 2, 4, 6, 8, 10, 15 and 20 weeks after fusion, almost 40% of the fused cultures lost their features received from parental cultures in the 4th week after fusion (Table 2). Pasha et al. (2007) tested stability of 3 fused cultures for 12 month and found that only one fused culture Viz., CP11 was stable after 12 month; another 2 cultures lost their stability during that

Table 2. Genotypic stability of the recombinant strains tested for a period of 20 weeks after protoplast fusion in YEPD slant for 20 weeks.

Stability of the fused culture	Weeks after fusion							
	0	2	4	6	8	10	15	20
FC 1	+	+	-	-	-	-	-	-
FC 2	+	+	-	-	-	-	-	-
FC 3	+	+	+	+	+	+	+	+
FC 5	+	-	-	-	-	-	-	-
FC 6	+	+	+	+	+	-	-	-
FC 8	+	+	+	+	+	+	+	-
FC 9	+	+	+	+	+	-	-	-
FC 10	+	+	+	+	+	+	+	-
FC 11	+	-	-	-	-	-	-	-
FC 12	+	+	+	+	+	+	+	+

*FC - Fused culture; (+) - Stable ; (-) - Not stable.

Table 3. DNA quantification of the parental and the selected recombinant strains using Nanodrop.

Yeast strain	<i>S cerevisiae</i>	<i>K. marxianus</i>	Fused culture 12
DNA content (ng/μl)	456.4 ± 19.02	571.4 ± 12.52	892.5 ± 15.68

period. The result of present investigation is similar to above findings. Out of the ten fused culture (except FC4 and FC7) studied for the biomass and ethanol production at 42 C using lactose as carbon source, fused cultures FC3 and FC12 were found to be stable for growth and ethanol production during the study period (20 weeks). Remaining 8 cultures lost the ability to utilize the lactose after 15 weeks (Table 2). Based on the fermentation ability and stability of the culture, FC 12 was taken for the further studies to confirm their stability and fermentation efficiency.

Recombinant characters of the fused cells

Genetic level confirmations of fusion were done by DNA quantification and RAPD. DNA content of the parent and fused culture were estimated using Nanodrop, the DNA content of parent 1, parent 2, fused culture were 456.4, 571.4 and 892.5 ng/ l of genomic DNA solution, respectively. The DNA content of the fused culture was higher than the both parents, because of the nuclear fusion that occurred between both the parents (Table 3). Tahoun et al. (1999) measured the DNA content of the parental and fused culture by using diphenylamine reagent method. Result showed that DNA content of the fused culture was higher than the parental cultures. The result of the present investigation was in accordance with the above finding.

RAPD was done for the further confirmation of fusion;

using 3 primers (OPQ2, OPQ4 and OPQ6) and the amplification pattern of the DNA fragments for the strains under investigation were obtained, as shown in Figure 2. The fused culture FC 12 was confirmed as nuclear fusion of both parental cultures, because of the complementary banding pattern of the parental strains. This is conformed with the findings of Francis and Clair (1993) who demonstrate that F1 hybrids showed bands characteristic of both parental strains.

Polypeptide profile of temperature tolerant strains by SDS-PAGE analysis

The polypeptide profile was seen with reference to protein marker ranging from 14.3 - 97 KDa. Lane 1 of 10% polyacrylamide shows the medium molecular weight marker, lane 2, 3, 4 were represent the protein profile of parent 1 exposed to different temperatures (35, 40 and 45 C). The parent 2 protein profiles and fused culture protein profile are lanes shown in 5, 6, 7, 8, 9 and 10. HSP 70 was seen in all the lanes of the parent 2 and fused culture (Figure 3) . In the present investigation, the higher thermotolerance of parent 2 and fused cultures were positively correlated with the synthesis of HSP 70 KD. It has already been reported that HSP 70 KD was involved in a variety of functions, such as helping newly synthesized proteins to assemble and fold correctly, denaturation of proteins (or prevention of denaturation) during a heat shock, facilitation of protein transport and

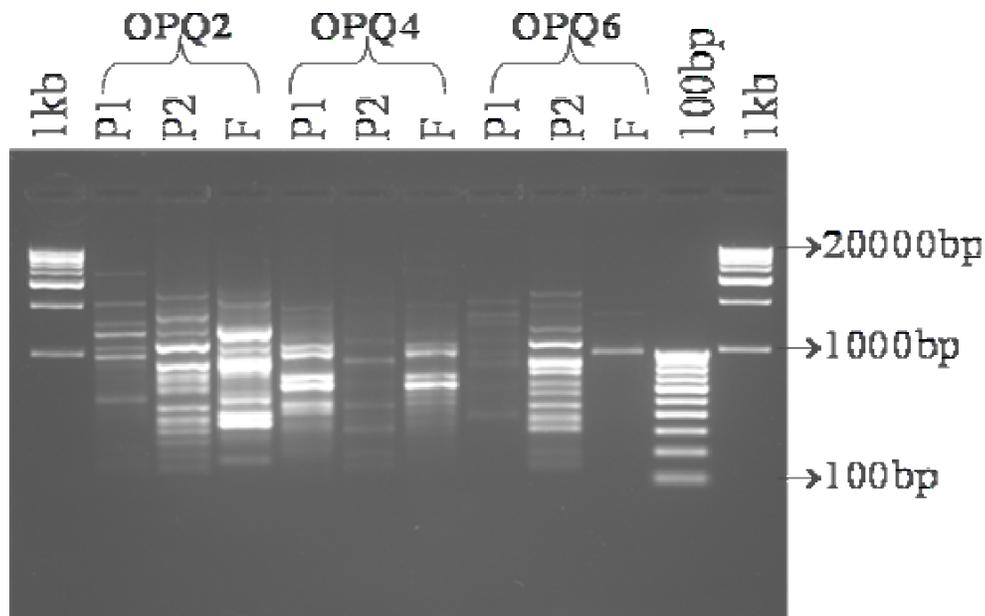


Figure 2. Random amplified polymorphic DNA of the parental and fused cultures.
 *OPQ2, OPQ4, OPQ6 – Primer, P1 - Parent 1 – *S. cerevisiae*, P2 - Parent 2 – *K. marxianus*, F - Fused culture 12.

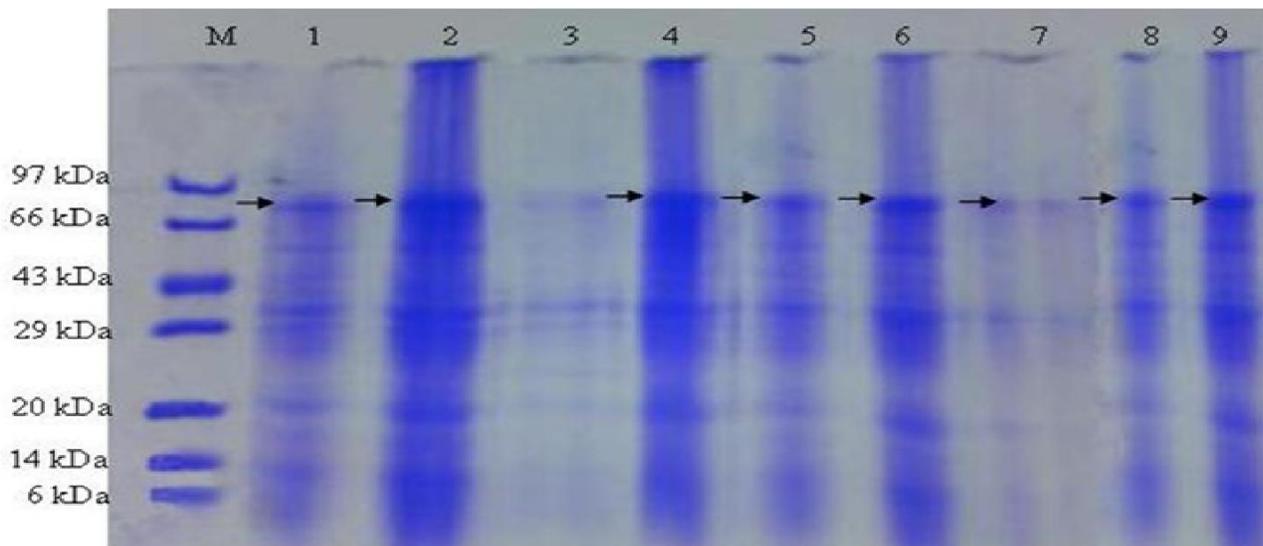


Figure 3. SDS-PAGE for parental and fused culture at different temperature.
 1- Parent 1 at 35°C, 2- Parent 1 at 40°C, 3 - Parent 1 at 45°C, 4- Parent 2 at 35°C, 5- Parent 2 at 40°C, 6 - Parent 2 at 45°C,
 7-Fused culture at 35°C, 8- Fused culture at 40°C, 9- Fused culture at 45°C.

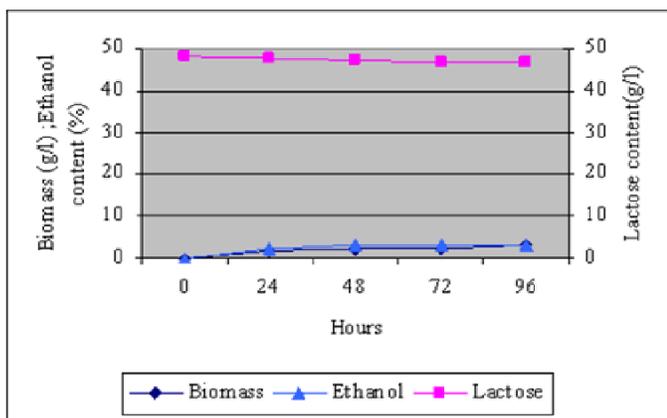
delivery in cell membrane system and promoting disassembly, dissociation and elimination of proteins (Hayes and Dice, 1996).

Substrate fermentation

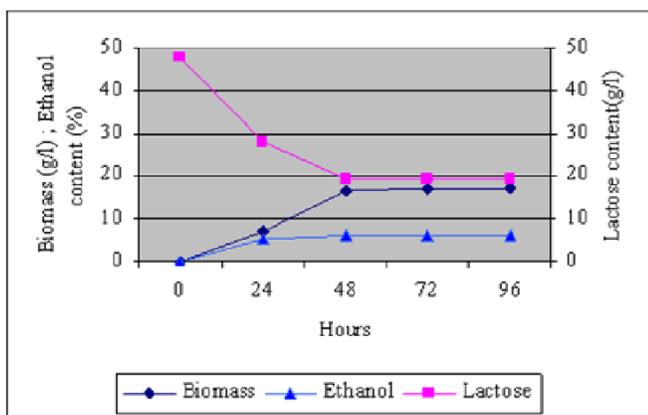
Fermentation was performed in 250 ml Erlenmeyer flask

containing 100 ml of CWM. The flasks were inoculated with 2% of inoculum obtained from 24 h old cultures of parental and fused culture and incubated at 42°C with 100 rpm/min. Samples were taken periodically from the CWM and analyzed for live cell population, biomass, lactose and ethanol concentration during fermentation and the results were shown in Figures 4a, b and c. The biomass yield and ethanol production was higher in the

4a. Parent 1



4b. Parent 2



4c. Fused culture 12

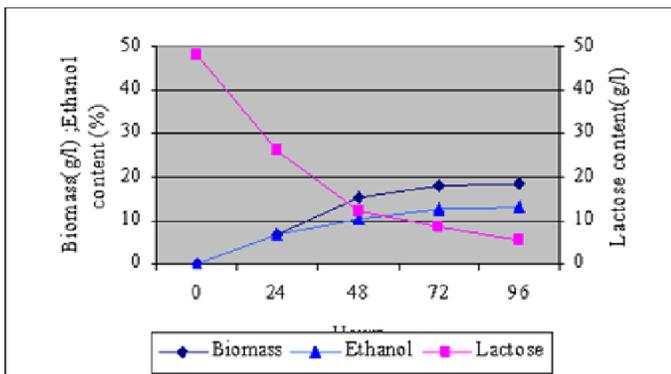


Figure 4. Growth and ethanol production of the parental and fused culture 4a Parent 1.

fused culture compared to the parental cultures. In 72 h of fermentation period, the fused culture produced the biomass of 18.09 g/lit with an ethanol yield of 12.5%, which was higher than the parental cultures (Figure 4c).

Kargi and Ozmihci (2006) found that ethanol concentration increased with time and reached a constant final concentration at the end of 72 h of incubation in CWP (Cheese whey powder) concentration between 50 and

150 g/l. Similar to sugar utilization, ethanol fermentation was slow for the first 72 h for sugar concentration above 100 g/l, probably due to osmotic pressure caused by high sugar concentrations. Ethanol production increased considerably after the first 72 h of adaptation period for sugar concentration above 100 g/l. The maximum ethanol concentration was 10.5% (V/V). Ethanol was obtained at the end of 216 h with initial sugar concentrations above 100 g/l slowed down ethanol formation; however, improved the final ethanol concentration considerably. Similarly, Pasha et al. (2007) showed the fused culture (CP 11) produced more biomass and ethanol than any of its parental cultures using xylose at 42 °C. More ethanol was produced by fused mutant CP 11 at 42 °C with xylose 150 g/l, Compared with parental strains. The amount of ethanol produced by parent VS3 strain was nil, whereas, CP11 strain produced 26.1 g/l ethanol at 42 °C. The fused yeast was found to be superior to the parent VS3 for ethanol production using lignocellulosic substrates.

Conclusion

Bioethanol production from waste is one of the preferable areas of alternate energy production. Protoplast fusion is an important tool for *gene* manipulation, because, it breaks down the barriers to genetic exchange imposed by conventional mating systems. Protoplast fusion technique has a great potential for genetic analysis and for strain improvement. This present study found that the fused culture FC12 is the efficient strain to produce high ethanol from the cheese industry waste (Whey). The strain developed has got the desirable characteristics such as having the high ethanol production, temperature tolerance and lactose utilizing capacity. This study has helped in developing a recombinant yeast strains by intergeneric protoplast fusion for, higher amount of ethanol production from waste with reduced cost for waste water treatment of whey for reduction of BOD

ACKNOWLEDGEMENT

The authors express their sincere thanks to Dr. D. Balachandar Associate Professor, Department of Agricultural Microbiology, TNAU and Dr. T. K. Raja, Retired Professor, VIT for their encouragement, spontaneous help and interest in the execution and refinement of this study.

REFERENCES

- Banat IM, Nigam P, Marchant R (1992). Isolation of thermo tolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. *World J. Microbiol. Biotechnol.*, 8: 259-263.
- Barnett JA, Payne W, Yarrow D (1986). "Yeasts: characteristics and identification", Cambridge University Press, Cambridge, New York, New Rochelle, Melbourne, Sydney.
- Dziuba E, Chmielewska J (2002). Fermentative activity of somatic

- hybrids of *saccharomyces cerevisiae* and *candida shehatae* or *pachysolen tannophilus*. Elec. J. Pol. Agri. Univ. Biotechnol., 5: 1-12.
- Farahnak F, Seki T, Ryu DY, Ogrzydziak D (1986). Construction of lactose-assimilating and high ethanol producing yeasts by protoplast fusion. Appl. Environ. Microbiol., 51: 362-367.
- Ferrari MD, Loperena L, Varela H (1994). Ethanol production from concentrated whey permeates using a fed-batch culture of *Kluyveromyces fragilis*. Biotechnol. Lett., 16: 205-210.
- Francis DM, Clair (1993). Outcrossing in the homothallic oomycete, *Pythium ultimum*, detected with molecular markers. Curr. Genet., 24: 100-106.
- Gunasekaran P, Raj KC (1999). Ethanol fermentation technology – *Zymomonas mobilis*. Curr. Sci., 77: 56-68.
- Hayes SA, Dice (1996) J F Roles of molecular chaperones in protein degradation. J. Cell Biol., 132: 255-258.
- Holzberg I, Finn RK, Steinkraus KH (1967). A kinetic study of the alcoholic fermentation of grape juice. Biotechnol. Bioeng., 9: 413-427.
- Kargi F, Ozmihci S (2006). Utilization of cheese whey powder for ethanol fermentation: Effects of operating parameters. Enzyme Microbial technol., 38: 711-718.
- Kosikowsk (1979). Whey utilization and whey products. J. Dairy Sci., 62: 1149-1160.
- Lin CCL, Fung DYC (1985). Effect of dyes on the growth of food yeasts. J. Food Sci., 50: 241-244.
- Lowry OH, Rose NJ, Farr AL (1951). Protein measurement with folin phenol reagent. J. Biol. Chem., 193: 265-275.
- Mahoney RR, Nickerson TA, Whitaker JR (1975). Application of acid lactase to wine making from cottage cheese whey concentrates. J. Dairy Sci., 58: 1620-1629.
- Martins C, Horii J, Pizzirani-Kleiner A (1999). Characterization of fusion products from protoplasts of yeasts and their segregants by electrophoretic karyotyping and RAPD. Revista De Microbiologia. 30: 71-76.
- Pasha CR, Kuhad C, Rao CV (2007). Strain improvement of thermo tolerant *Saccharomyces cerevisiae* VS3 strain for better utilization of lignocellulosic substrates. J. Appl. Microbiol., 103: 1480-1489.
- Petit J, Boisseau P, Arveiler B (1994). Glucanex: a cost - effective yeast lytic enzyme. Trends genet., 10: 4-5.
- Saumya B, Hemchick PH (1983). Simple and rapid method for disruption of bacteria for protein studies. Appl. Environ. Microbiol., 46: 941-943.
- Sing D, Nigam P, Banat IM, Marchant R, Mchale AP (1998). Review: Ethanol production at elevated temperatures and alcohol concentrations: Part II – Use of *Kluyveromyces marxianus* IMB3. World J. Microbiol. Biotechnol., 14: 823-834.
- Sing MA, S Lindquist (1998). Thermo tolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. Trends Biotechnol., 16: 460-468.
- Svitree L, Sumpradit T, Kitpreechavanich V, Tuntirungkij M, Seki T and Yoshida T (2000). Effect of Acetic Acid on Growth and Ethanol Fermentation of Xylose Fermenting Yeast and *Saccharomyces cerevisiae*. Kasetsart J. (Nat. Sci.), 34 : 64-73.
- Tahoun MK, Nemr T M, Shata OH (1999). Ethanol from lactose in salted cheese whey by recombinant *Saccharomyces cerevisiae*. Z. Lebensm. Unters. Forsch. a. 208: 60-64.
- Van der Walt JP (1970). "The yeasts. A taxonomic study". 2nd edition, North-Holland Publishing Company, Amsterdam. pp. 34-113.