Indian J. Microbiol. (December 2007) 47:310–316

ORIGINAL ARTICLE

Optimization of fermentation parameters for production of ethanol from kinnow waste and banana peels by simultaneous saccharification and fermentation

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Received: 15 June 2007 / Final revision: 13 August 2007 / Accepted: 5 October 2007

Abstract A study was taken up to evaluate the role of some fermentation parameters like inoculum concentration, temperature, incubation period and agitation time on ethanol production from kinnow waste and banana peels by simultaneous saccharification and fermentation using cellulase and co-culture of *Saccharomyces cerevisiae* G and *Pachysolen tannophilus* MTCC 1077. Steam pretreated kinnow waste and banana peels were used as substrate for ethanol production in the ratio 4:6 (kinnow waste: banana peels). Temperature of 30°C, inoculum size of *S. cerevisiae* G 6% and (v/v) *Pachysolen tannophilus* MTCC 1077 4% (v/v), incubation period of 48 h and agitation for the first 24 h were found to be best for ethanol production using the combination of two wastes. The pretreated steam exploded biomass after enzymatic saccharification containing 63 gL^{-1} reducing sugars was fermented with both hexose and pentose fermenting yeast strains under optimized conditions resulting in ethanol production, yield and fermentation efficiency of 26.84 gL^{-1}, 0.426 gg^{-1} and 83.52 % respectively. This study could establish the effective utilization of kinnow waste and banana peels for bioethanol production using optimized fermentation parameters.

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Keywords Kinnow waste · Banana peels · SSF · Ethanol · Fermentation parameters · Cellulase production

Introduction

With the inevitable depletion of world's energy supply, there has been an increasing interest worldwide in alternative sources of energy[1,2,3]. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars and used as a partial gasoline replacement in a few countries in the world. Ethanol production through fermentation may provide an economically competitive source of energy [4,5] by its incorporation into gasoline. Among the crucial factors affecting ethanol fermentation, culture conditions play significant role on growth of yeast as well as ethanol production. A lot of work is being carried out in the area of ethanol production from lignocellulosics such as forestry wastes, corn stalk and cobs, wheat straw, grasses, rice straw etc., but only a limited literature is available on ethanol production from banana and citrus waste[6,7,8]. Citrus and banana are important fruit crops of the world and India alone contributed to 24 and 8.5% of the total world production of banana and citrus respectively in the world [9]. Both banana and citrus leave a sizeable amount of residues after processing in the form of peels; peels, seeds and pulp respectively. Such residues pose considerable disposal problems and ultimately leads to environmental pollution. Kinnow (*Citrus reticulata*), a hybrid between "King and Willow leaf" belongs to the citrus family is an important fruit of North India and is extensively grown in Punjab, Haryana, parts of Himachal Pradesh and Rajasthan. The production of kinnow in India is estimated to be around 0.4 million tones[10]. Kalra *et al*.[11] reported that kinnow residues

are rich in carbohydrates and account for about 55-60% of the weight of the raw fruit. Both residues, being rich in carbohydrates, crude proteins, and reducing sugars[8,11] can serve as potential feedstock for ethanol production. Most of the available literature suggests the use of either recombinants or co-cultures of hexose and pentose fermenting yeasts with enzymes for ethanol production from lignocellulosics. Therefore, citrus waste and banana peels as substrates can serve as an affordable and renewable low cost raw material for bioethanol production. The advantage of simultaneous saccharification and fermentation (SSF) is that a multistage process for the production of ethanol is carried out in one reactor and glucose produced during saccharification is simultaneously fermented to ethanol by yeast cells [12]. Besides, being relatively cheaper as compared to the process involving external addition of enzymes, this process also avoids catabolite repression of the enzymes produced during saccharification step due to higher glucose concentration. Certain fermentation parameters such as inoculum, enzyme and substrate concentration besides optimum pH, temperature, time, agitation etc play an important role in obtaining good ethanol yield. Thus, the present study was planned with the objective of optimizing the fermentation parameters like inoculum concentration, temperature, incubation period and agitation time using kinnow waste and banana peel through SSF for maximizing ethanol production using co-cultures of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* respectively.

Materials and Methods

Trichoderma reesei Rut C-30 used for cellulase production and *Saccharomyces cerevisiae* G were procured from the culture collection centre, Department of Microbiology, Punjab Agricultural University, Ludhiana, India and *Pachysolen tannophilus* MTCC 1077 was obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. Fungal culture was maintained on PDA and yeast cultures was maintained on Glucose Yeast Extract Agar (GYEA) and both were stored at 4 ± 1°C and subcultured fortnightly.

Substrate and pretreatment: Kinnow waste (peel + segment membranes + seeds) and banana peels were obtained from a local fruit processing plant at Ludhiana, India. The substrate was washed 2-3 times with sterile water to remove extraneous matter and dried in oven at 70°C to a constant weight. Oven dried substrate was then ground to a particle size corresponding to 40 mesh using electrical grinder. Both the residues were mixed in the ratio 4:6 (Kinnow waste: banana peel) which was used as a substrate for fermentation. In one of our experiments , results of which are reported elsewhere, it was observed that out of different combinations tried, the ratio of 4:6 (kinnow waste: banana peel) using steam treatment resulted in release of maximum amount of sugars from the biomass and thus this ratio was used for further trials. Powdered substrate was subjected to steam depressurization prior to SSF. The experiment was performed in a vertical autoclave at 15 psi for 1.0 h followed by sudden depressurization by fully opening the steam exhaust valve of autoclave with the objective of obtaining the maximum quantity of fermentable sugars using least pretreatment time. Sharma *et al*. [13] have reported use of steam explosion treatment at 15 psi for 1.5 h for sunflower stalks as optimum for enzymatic hydrolysis. The. Solid : liquid ratio during steam depressurization was maintained at 1:8.

Enzyme production: Cellulase was produced by *Trichoderma reesei* Rut C-30 under submerged batch conditions using Chahal and Gray modified basal synthetic medium[14] supplemented with 1% cellulose. One hundred milliliter of basal medium was dispensed into 250 mL Erlenmeyer flasks containing 1 g cellulose. The flasks were autoclaved at 15 psi for 20 min, cooled to room temperature and inoculated with 10 mL of fungal culture pre grown on GYE medium. Flasks were then placed on a rotary shaker (150 rpm) at 28°C for 10 days. The samples were drawn at 24 h interval from fourth day onward and analysed for filter paper activity, CMCase and cellobiase [15]. The samples were centrifuged at 6,000 rpm at 5^0C for 10 min (Eltek Mp 400 R, India) and the supernatant was analysed for the enzyme activity as mentioned above.

Enzymatic saccharification: 100 mL of steam exploded material (residue and hydrolysate) in 250 mL Erlenmeyer flasks was subjected to enzymatic saccharification using the crude filterate at 4 FPU g^{-1} prepared from *T. reesei* RC 30. The flasks were incubated at 30^0C for 72 h on an incubator shaker to observe for the saccharification potential of the enzyme.

Simultaneous Saccharification and Fermentation (SSF): 100 mL of steam exploded material containing both residue and hydrolysate produced from 25 g of biomass in 500 mL Erlenmeyer flasks was supplemented with 3.0 g yeast extract and 3.0 g peptone L^{-1} and used as a basal fermentation medium. The medium was sterilized at 15 psi at 121^0C for 20 min. Since, the enzyme was used @ 4FPU g^{-1} (FPU), the required volume of enzyme along with both *S. cerevisiae* and *P. tannophilus* cultures was added aseptically into the sterilized flasks. As has been reported in the literature[16] that steam explosion of cellulosic biomass results in the release of most of the pentose sugars of which xylose is the major constituent *P. tannophilus* was included as *S. cerevisiae*

selected in this study is able to convert only hexose sugars and not pentose sugars into ethanol. The optimization experiments were carried out by varying inoculum concenntration, temperature, pH, incubation period and agitation time to optimize the most ideal conditions for maximizing ethanol production, however the enzyme concentration was standardized in terms of Filter Paper activity and the enzyme was used at 4FPU g^{-1}dry substrate as mentioned above in all experiments.

Fermentation parameters for ethanol production: *Inoculum preparation* : The freshly prepared yeast cultures of *S. cerevisiae* and *P. tannophilus* on agar slants were inoculated into 100 mL GYE broth in 250 mL Erlenmeyer flasks and incubated at 30 ± 2ºC for 24 h on a rotary shaker. A cell count of about 3.6 x 10^8 and 2.1 x 10^8 cell mL^{-1} respectively was obtained after 24 h and the same were used as inoculum for future experiments.

Effect of operational parameters: The effect of inoculum size on ethanol fermentation by the process of SSF using mixed cultures of *S. cerevisiae* and *P. tannophilus* was carried out by varying the inoculum concentration of both *S. cerevisiae* and *P. tannophilus* from 2 to 10 % (v/v). Effect of temperature on ethanol fermentation by the process of simultaneous saccharification and fermentation using mixed cultures of *S. cerevisiae* and *P. tannophilus* was carried out by varying the temperatures to 20, 24, 28, 30, 32 and 35ºC and temperatures were established only at the beginning of the experiment. Effect of incubation period and agitation time on ethanol fermentation was carried out by varying the incubation period and agitation time from 12 to 96 h.

Analytical methods: Cellulose content was determined by the method described in the literature[17]. Hemicellulose was determined by the method described by Goering and Vansoest [18]. Total sugars and reducing sugars were determined by following the standard procedures [19,20]. Cellulase was produced by *Trichoderma reesei* Rut C-30 under submerged batch conditions using Chahal and Gray modified basal synthetic medium supplemented with 1% pure cellulose. Ethanol was estimated using GC (CIC, Baroda, India). The fermentation efficiency was calculated as:

> Ethanol produced/Theoretical maximum ethanol yield from sugar x 100
> Theoretical maximum ethanol yield = 0.51 g ethanol per gram sugar.

All the experiments related to fermentation parameters were carried out in triplicate in simple CRD and the data was analysed using the cpcs software procured from the Department of Mathematics and Statistics, Punjab Agricultural University, Ludhiana, India as described earlier by Oberoi *et al*. [21]

Results and Discussion

Chemical composition of citrus waste and banana peels without pretreatment: The moisture content was found to be 73.4 and 79.2% in citrus waste and banana peels respectively. Kinnow waste and banana peels on dry weight basis contained cellulose (23.48 and 28.67%), hemicellulose (20.54 and 18.40%), total sugars (79.10 and 92.88 mg g^{-1}) and reducing sugars (23.07 and 36.83 mg g^{-1}) respectively (data not shown).

Enzymatic saccharification of biomass: The Filter paper activity, CMCase and cellobiase activity were found to be 1.01, 1.16 and 0.11 U mL^{-1} respectively was found to be maximum on the 8th day of incubation and hence the crude filterate extract from the flasks obtained on the 8th day was centrifuged, filtered through 0.45µ millipore filters using filtration assembly (Millipore) and was used for SSF experiments along with the inoculum . As mentioned earlier, the enzyme addition was based on Filter paper activity only and thus was standardized accordingly depending upon the activity observed. Fig 1. indicates the saccharification of the banana peel and kinnow waste mixture at different time intervals using the crude filterate of the enzyme. It is clear from the Figure that the maximum saccharification resulting in maximum quantity of total reducing sugars was observed after 48 h of incubation and beyond which the sugar concentration remained constant or showed a slight fall mainly due to feedback inhibition or catabolite repression. Our results are in harmony with the results reported earlier [6].

Effect of cell concentration (inoculum size) of yeasts: Fig 2 indicates the varying inoculum concentration of *P. tannophilus* and fixed concentrations of *S. cerevisiae*. The amount of sugar consumed and ethanol produced increased linearly with increase in initial cell concentration from 2.0 to 10% with 6% *S. cerevisiae* G (v/v) and 4% *P. tannophilus* MTCC 1077 (v/v) inoculum concentration producing maximum ethanol yield of 0.394 gg^{-1}. Increasing the inoculum concentration beyond the above mentioned concentration resulted in decline in fermentation yields which is in accordance with the results reported earlier[22].

Effect of temperature: The results in Fig 3. indicate that maximum ethanol yield of 0.376 gg^{-1} was produced at temperature of 30^0C. The ethanol yield increased with the increase of temperature from 20 to 30^0C up to 48 h of

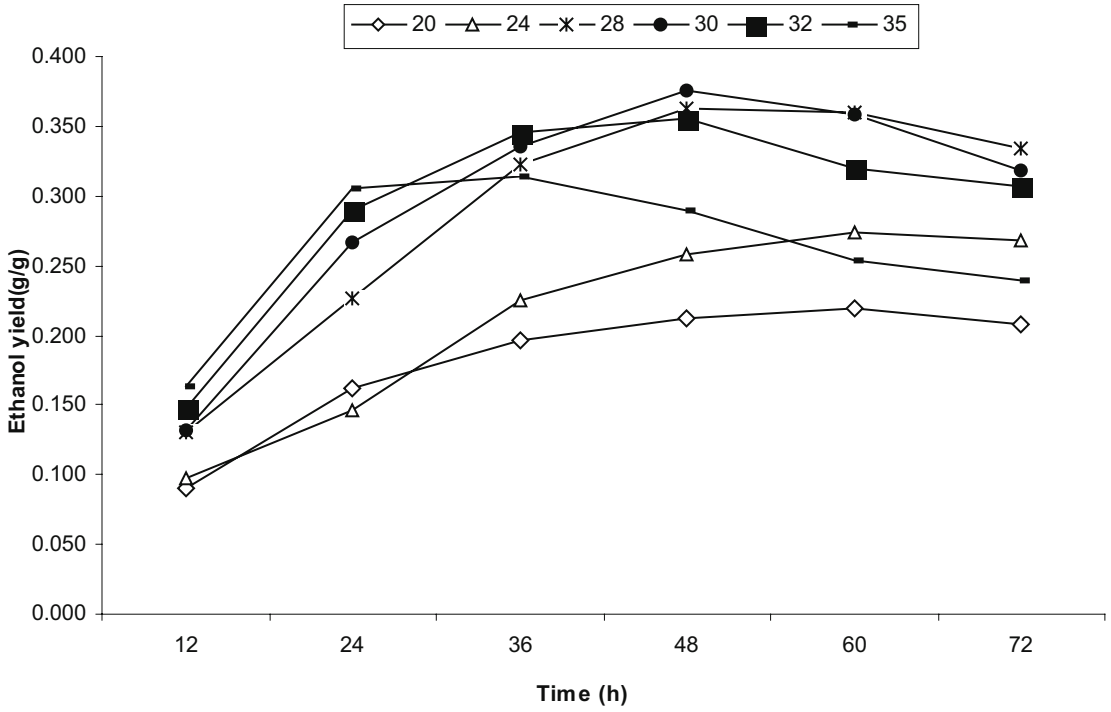

Fig. 1 Effect of enzymatic hydrolysis of citrus waste and banana peels.

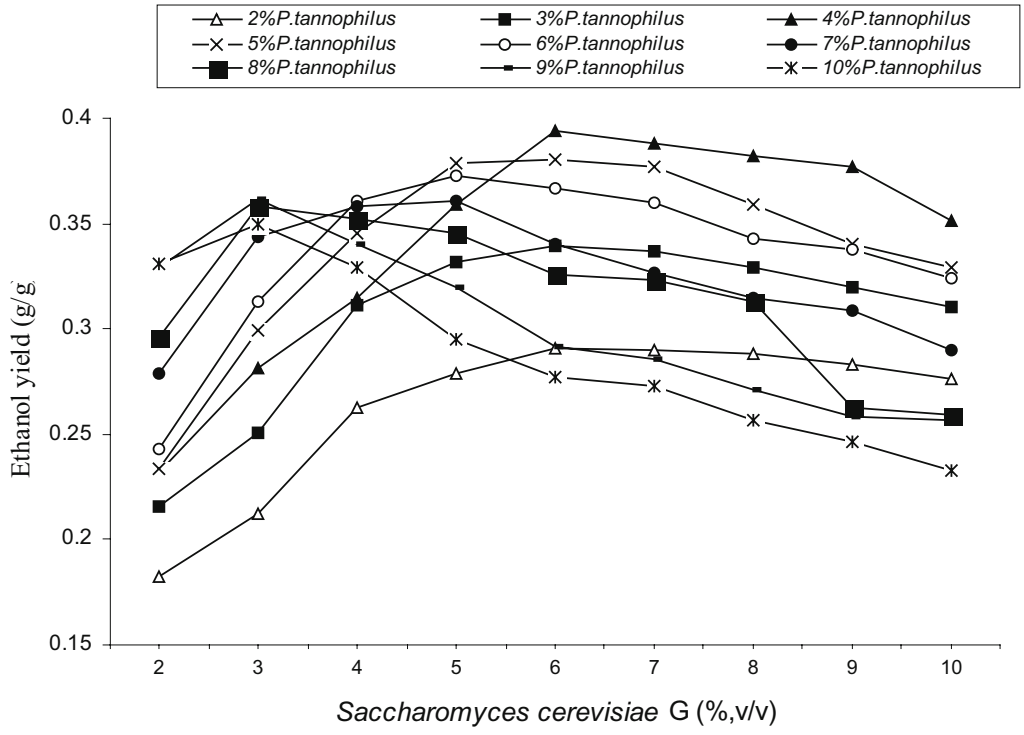

Fig. 2 Effect of different inoculum concentrations of *Pachysolen tannophilus* MTCC-1077 and *Saccharomyces cerevisiae* G for ethanol.

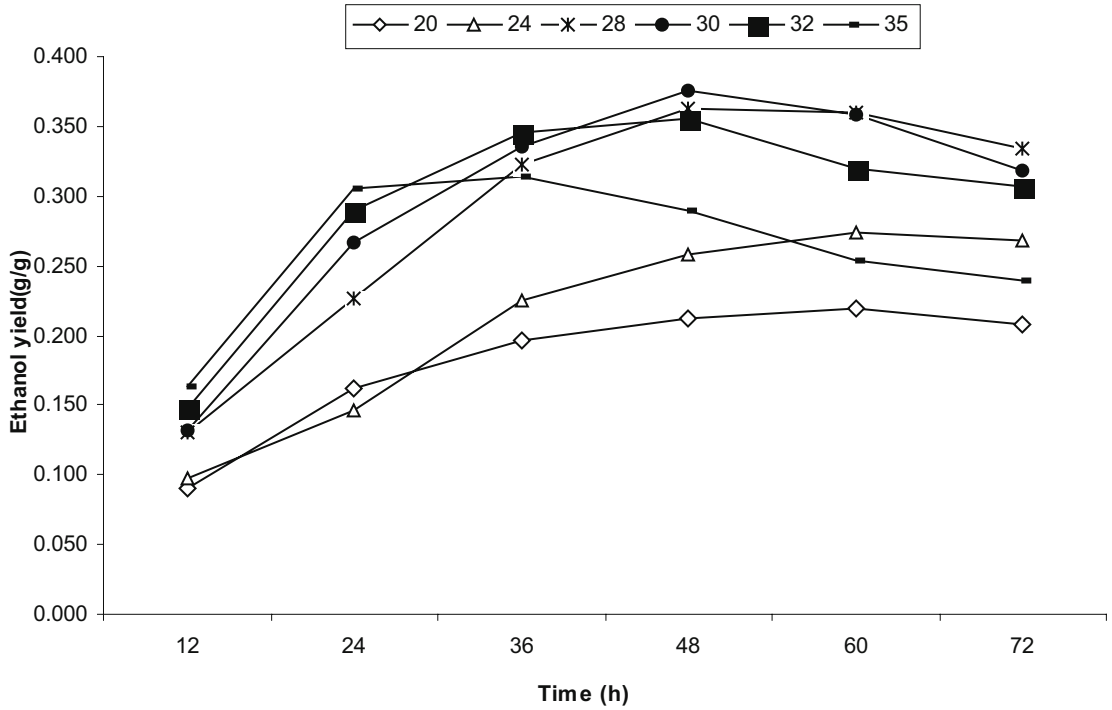

Fig. 3 Effect of different temperature and incubation period on ethanol yield.

incubation after which it declined (Fig.3, Table 1). However, temperatures beyond 30^{0}C showed a fall in ethanol production which is in line with the findings of El-Refai *et al*. [23] who reported maximum ethanol productivity from beet molasses by *S. cerevisiae* Y-7 after 48 h of incubation at 30°C. Verma et al [24] also reported 30°C as the optimum temperature for maximum ethanol production using starch employing co-culture of amylolytic yeast and *S. cerevisiae*. Thus, optimum temperature for simultaneous saccharification and fermentation of kinnow waste and banana peels was found to be 30°C with maximum ethanol yield of 0.376 gg^{-1} and fermentation efficiency of 74.11 % (Table 1) at 48 h of incubation. At temperatures lower or higher than optimum, less ethanol production was observed. Decline in ethanol yield at increased temperature might be due the inactivation of enzymes involved in ethanol production pathways. These observations are consistent with findings of other authors [25,26]. Therefore the future experiments were conducted at incubation temperature of 30^{0}C.

Effect of Incubation period: Co-culture of *S. cerevisiae* G and *P. tannophilus* MTCC 1077 along with enzymes exhibited maximum ethanol yield (0.398 gg^{-1}) at 48 h of incubation (Table 2). In a similar study carried out on effect of incubation period on ethanol productivity, Wright [27] reported the maximum ethanol production of 4% (w/v) while converting the wheat straw to ethanol after 48 h of incubation employing process of simultaneous saccharification and fermentation using *T. reesei* cellulase and *Kluyveromyces fragilis*. Sharma [28] has reported maximum ethanol yield and fermentation efficiency of 0.397 gg^{-1} and 77.84 per cent, respectively after 36 h of incubation at 30°C using mixed culture of *S. cerevisiae* and *P. tannophilus*. Some authors have reported maximum ethanol yield after 48 h of incubation from starchy materials [29,30].

Table 1 Effect of different temperatures on ethanol production at 48 h of incubation.

Temperature (°C)	Ethanol production (g L^{-1})	Ethanol yield (g g^{-1})	Fermentation Efficiency (%)
20	14.26	0.226	44.31
24	17.84	0.283	55.49
28	23.42	0.371	72.74
30	23.79	0.378	74.11
32	22.67	0.359	70.39
35	20.18	0.320	62.80
C.D (0.05)	0.227	0.0013	

Effect of agitation time: Data in Table 3 shows the effect of agitation time on ethanol production. It is clear from the data that lower agitation time was found to be suitable for

Table 2 Effect of incubation period on ethanol production.

Time (h)	Ethanol conc.(g L^{-1})	Ethanol yield (g g^{-1})	Fermentation efficiency (%)
12	11.64	0.185	36.27
24	20.76	0.330	64.71
36	23.68	0.376	73.73
48	25.10	0.398	78.04
60	24.90	0.395	77.45
72	24.39	0.387	75.88
C.D (0.05)	0.106	0.0032	

Table 3 Effect of agitation time on ethanol production.

Agitation Time (h)	Ethanol conc.(g L^{-1})	Ethanol yield (g g^{-1})	Fermentation Efficiency (%)
6	21.49	0.341	66.86
12	23.68	0.376	73.72
24	25.08	0.398	78.04
36	25.00	0.397	77.84
48	24.76	0.393	77.06
60	24.40	0.387	75.88
72	23.81	0.378	74.18
C.D (0.05)	0.119	0.0054	

ethanol production. The highest ethanol yield and fermentation efficiency of 0.398gg^{-1} and 78.04% respectively were observed with 24 h of agitation which is primarily due to initial oxygen requirements of yeast cells. Excess oxygen in the fermentation medium could lead to increased cell growth at the cost of ethanol productivity [31]. However, Kosaric et al [32] have confirmed that some oxygen is required by fermenting yeast for production of polyunsaturated fats and lipids.

Ethanol production through optimized parameters : There was a continuous increase in ethanol production, ethanol yield and fermentation efficiency from 12 to 48 h of incubation using the optimized conditions (Table 4). A slight fall in all the parameters was observed with increase in fermentation time beyond 48 h. The highest ethanol yield and fermentation efficiency of 0.426 g g^{-1} and 83.52 %, respectively were observed at 48 h of incubation. El-Refai *et al*. [23] attained maximum fermentation efficiency of 83.3 % from acid treated beet molasses after 48 h incubation at 30°C using *S. cerevisiae* Y-7. Similarly, El-Abayad et al [26] have reported maximum fermentation efficiency of 77% after 48 h while fermenting beet molasses by *S. cerevisiae* Y-7. However, Sharma [28] have reported the maximum ethanol yield and fermentation efficiency of 0.397 g g^{-1}

Table 4 Ethanol production through optimized parameters using SSF.

Time (h)	Ethanol production (gL^{-1})	Ethanol yield (gg^{-1})	Fermentation Efficiency (%)
12	11.87	0.188	36.86
24	21.97	0.348	68.23
36	24.24	0.384	75.29
48	26.84	0.426	83.52
60	26.16	0.415	81.37
72	25.22	0.400	78.43
CD (0.05)	0.167	0.0081	

and 77.84 %, respectively after 36 h of incubation at 30°C using mixed culture of *S. cerevisiae* and *P. tannophilus*. These results suggest that various fermentation parameters drastically influenced the production of ethanol by co-culture of *S. cerevisiae* G and *P. tannophilus* MTCC 1077 to a large extent. This study could establish the optimized fermentation parameters for effective utilization of banana peels and kinnow for ethanol production.

Conclusion

This study could establish that both kinnow waste and banana peel which have not been exploited commercially for any industrial application and are poorly disposed could effectively be used for ethanol production through the process of simultaneous saccharification and fermentation. The process with optimized fermentation parameters described in the paper could be used for scaling up of the process to a pilot scale or commercial fermenter level thereby making the process more cost effective.

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