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Hydrothermal and Enzymatic Pretreatment of Apple Pomace for Bioethanol Production by Solid-state Fermentation

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ABSTRACT: Apple pomace (AP) is agro-industrial lignocellulosic waste generated after extraction of juice from fresh apples. The main objective of this study was, pretreatment of apple pomace with reduced energy input and use of pretreated apple pomace in bioethanol production. In this study, dried apple pomace was pretreated using hydrothermal and enzymatic techniques. Combination of both pretreatments produced highest amount of reducing sugars i.e. 43.1 g/100 g dry matter (DM), which is 10.5 times higher than the initial level of reducing sugars (4.1 g/100 g DM) present in apple pomace. Pretreated apple pomace was further used as substrate in solid-state fermentation for ethanol production. Under optimized fermentation conditions using *S. cerevisiae* with co-culture (isolate APW-12), 17.5 g ethanol/100 g of dried apple pomace was obtained. In conclusion, high amount of reducing sugars were obtained from apple pomace with minimum use of chemicals, water and energy using hydrothermal and enzymatic process. Also, the dried apple pomace which has longer shelf-life can be used for producing significant amounts of ethanol.

Keywords: apple pomace, pretreatment, *Saccharomyces cerevisiae*, co-culture, solid-state fermentation, ethanol production.

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INTRODUCTION

Apple is a fruit with over 30 species belonging to the genus *Malus* and is mainly grown in temperate regions across the world (Magyar *et al.*, 2016; Joshi and Devrajan, 2008). Processing of apple for production of ready-to-serve apple juice, wine, cider, apple juice concentrate, purees, jams etc. generates apple pomace as a waste which accounts for about 25% of fresh apple (Shalini and Gupta, 2010). Apple pomace comprises of skin, seeds and pulp and has high amount residual sugars and polysaccharides, but it is simply dumped in landfills or is converted to vermicompost and is prone to microbial growth and spoilage causing environmental pollution. AP has been used as animal feeds but has low nutritional value because of its low protein content. This agro-industrial waste has high biological oxygen demand and should be disposed-off in environment friendly way (Magyar *et al.*, 2016). This led to the idea of generating valuable product like bioethanol and simultaneously dealing with the problem of indiscriminate dumping of apple pomace out in the open by fruit processing plants.

A few studies have used apple pomace for production of bioethanol like, Hang *et al.* (1981) carried out solid state fermentation without any pretreatment and reported approximately 4.3 g ethanol/100 g of wet apple pomace. Nogueria *et al.* (2005) carried out alcoholic fermentation of aqueous extract of the apple pomace and were able to produce 7.3 g/L ethanol. Joshi and Devrajan (2008) performed solid-state fermentation by using sequential co-culture of five different yeast and produced 9.84% (v/w) ethanol. Parmar and Rupasinghe (2013) reported 19 g ethanol/100 g of dry matter after enzymatic and dilute acid pretreatment of apple pomace. Recently, Magyar *et al.* (2016) carried out ethanolic fermentation at industrial scale by chemical and enzymatic hydrolysis of apple pomace using *S. cerevisiae* and were able to produce 53.8 g/L ethanol. Apple pomace is a lignocellulosic biomass having a complex structure composed of homo and heteropolysaccharides. Pretreatment is required to loosen this rigid structure of polysaccharides and release the fermentable sugars. Using a pretreatment which is inexpensive and less energy consuming on a substrate which in turn is a waste, low cost and inedible, one can reduce the initial expenditure involved in production of bioethanol.

The main aim of this study was to optimize the pretreatment of apple pomace for releasing maximum reducing sugars possible and to use these sugars in bioethanol production using single and co-culture.

MATERIALS AND METHODS

A. Raw materials, microorganisms and media

Fresh apple pomace was obtained from Himachal Pradesh Horticultural Produce Processing and Marketing Corporation (HPMC), processing plant at Parwanoo, Himachal Pradesh, India.

It was dried in hot air dehydrator at 60ºC and was ground to a fine powder by using a hand operated disk-mill. The powder was stored in air tight containers at a cool and dry place for further use. *Saccharomyces cerevisiae* MTCC 173 was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. Isolate APW-12 belonging to the genus *Actinomyces* was isolated from soil in laboratory and is known to have cellulase activity (490 U/mL/min), xylanase activity (601 U/mL/min) and capacity to produce ethanol. YEPD medium (Yeast Extract 1%, Peptone 2% and Dextrose 2%, pH 6.0) and Nutrient medium (peptone 0.3%, beef extract 0.5%, sodium chloride 0.5%, pH 7.0) were used to cultivate and maintain the microorganisms. Industrial pectinase enzyme (Trizyme-5000) was obtained from Kaypeeyes Biotech (P) Ltd. Mysore, Karnataka, India.

B. Pretreatment of apple pomace

Hydrothermal pretreatment of AP was performed at 100ºC and 121ºC for 30-120 min. During pretreatment at 100ºC, four sets of sealed conical flasks containing AP powder (re-moistened to 70.89% w/w moisture) were kept in boiling waterbath for a duration of 120 min. After every 30 min flasks were removed and analyzed for the amount of reducing sugars present in it by DNS method (Miller, 1959). Pretreatment at 121ºC was carried out using an autoclave and similar procedure was followed as stated for pretreatment at 100ºC. The hydrothermal pretreatment condition which released maximum amount of reducing sugars with less energy consumption was selected for use in further experiments.

In enzymatic pretreatment, industrial pectinase enzyme (Trizyme-5000) was used for hydrolysis of apple pomace. Two sets of enzymatic pretreatments were performed, one where AP powder (re-moistened to 70.89% w/w moisture level) was treated with enzyme alone (EP-I) and other where enzyme treated AP was subjected to hydrothermal pretreatment (EP-II). After pretreatment reducing sugars released in each case were estimated by DNS method. Enzyme solution was prepared in 0.1M sodium acetate buffer (pH 5.0). Enzymatic pretreatment was optimized using variables such as; temperature (30-60ºC), enzyme concentration (50-400 g/L), reaction time (30-120 min) and substrate concentration (1-5 g). The pretreatment condition which released maximum reducing sugars with less energy consumption was selected for use in subsequent experiments.

C. Ethanolic fermentation using apple pomace as substrate

Inoculum Preparation. *Saccharomyces cerevisiae* MTCC 173 was used in all the fermentation experiments. Seed cultures were prepared in 250 mL Erlenmeyer flasks containing YEPD medium (yeast extract 1%, peptone 2% and dextrose 2%) which was inoculated with 24 h old *S. cerevisiae* culture with 0.2 optical density (OD_{600}). The flasks were kept in shaking incubator at 30ºC and 150 rpm for 24 h. Optical density (OD_{600}) of seed culture was adjusted to 2.0 with fresh YEPD broth and used in further experiments. Similar procedure was followed for the isolate APW-12 where, seed culture was prepared in nutrient broth medium.

Solid-state fermentation. Ethanol fermentation was optimized on the basis of following parameters: relative initial moisture in substrate (10-100% w/w), temperature (25-40ºC), *S. cerevisiae* inoculum size (1-10% v/v), co-culture (APW-12) inoculum size (1-10% v/v) and nitrogen source (1-5% v/v). During the optimization of first parameter (relative initial moisture in substrate), the amount of moisture initially present in fresh apple pomace (70.89% w/w) was presumed to be 100% relative moisture. Dry apple pomace was moistened with appropriate amount of water to obtain 10-100% relative moisture.Initial pH of AP was adjusted to 6.0. Re-moistened AP was subjected to optimized pretreatment. Pretreated AP was inoculated with 1% (v/v) inoculum of *S. cerevisiae* and fermentation was carried out at 30ºC for 72 h. Ethanol produced in each fermentation was estimated by potassium dichromate method (Caputi *et al.,* 1968) and reducing sugars were estimated by DNS method (Miller, 1959). The condition which produced maximum amount of ethanol was used in subsequent experiments.

RESULTS AND DISCUSSION

A. Hydrothermal pretreatment of apple pomace

Hydrothermal pretreatment of apple pomace was performed at 100ºC and 121ºC. The results of hydrothermal pretreatment are shown in Fig 1. The initial amount of reducing sugars in apple pomace was 0.041±0.015 g/g DM. The maximum amount of reducing sugars (0.052±0.059 g/g DM) was obtained after pretreating apple pomace at 100ºC for 2 h. On the other hand, heating apple pomace at 121ºC for 30 min, released 0.062±0.015 g/g DM of reducing sugars. In the next 90 min, approximately two times increase in reducing sugar level (0.102±0.047 g/g DM) was observed as compared to the amount obtained in first 30 min of heating apple pomace at 121ºC. Although, this achievement came with three times more energy consumption as compared to pretreatment at 121ºC for 30 min. Observing the increase in amount of reducing sugars to the amount of energy consumed, the optimum duration for hydrothermal pretreatment at 121ºC was recorded as 30 min. Bensah *et al.* (2015) performed hydrothermal pretreatment of elephant grass at 121ºC for 30 min at 6% solid loading and reported an increase of 6.2 g/100 g DM for glucan and 1.9 g/100 g DM for xylan when compared with untreated biomass.

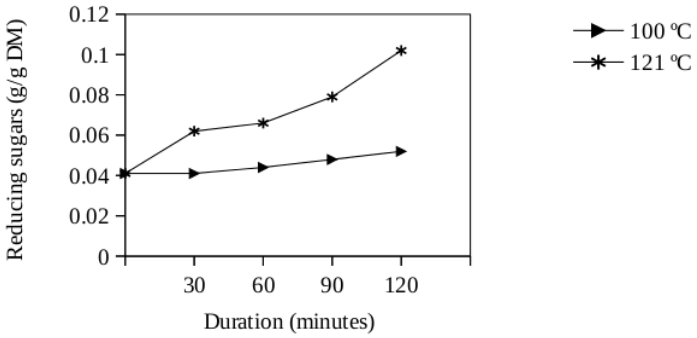

Fig. 1. Reducing sugars released from apple pomace after hydrothermal pretreatment at 100ºC and 121ºC for different time durations. The reducing sugars present in untreated control were 0.041±0.015 g/g DM.

Apiwatanapiwat *et al.* (2013) reported hydrothermal pretreatment of dry cassava pulp at 100ºC and 120ºC for 10 min and before carrying out hydrothermal pretreatment, 5 mL distilled water was added to one gram of dry cassava pulp. This process resulted in 70% saccharification after boiling the biomass at 100ºC, while 90% saccharification was achieved after autoclaving.

B. Enzymatic pretreatment of apple pomace

Two sets of enzymatic pretreatment were performed, one where AP was treated with enzyme alone (EP-I) and other where enzyme treated AP was subjected to hydrothermal pretreatment at 121ºC for 30 min (EP-II), the results are shown in Figure 2. During optimization of temperature for enzymatic pretreatment, maximum reducing sugars (RDS) were obtained at 50ºC and reaching to 0.081±0.034 g/g DM for EP-I and 0.129±0.031 g/g DM for EP-II (Fig. 2A). Magyar *et al.* (2016) in a similar way investigated the conversion of apple pomace waste to ethanol at industrial relevant conditions. In this study, enzymatic hydrolysis was performed at 50ºC using three enzymes i.e. cellulase (Ctec3), hemicellulase (Htec3) and pectinase (Pectinex) by maintaining pH 4.8 and 250 rpm for 24 h and reported 57.5 and 50.1 g/L glucose and fructose release respectively.

Optimized temperature was used in the next experiment, where 300 g/L enzyme concentration resulted in RDS i.e. 0.159±0.021 g/g DM for EP-I and 0.178±0.020 g/g DM for EP-II (Fig. 2B). Gama *et al.* (2017) in a similar type of study used an artificial neural network to predict the optimal conditions for enzymatic hydrolysis of apple pomace and as per their observation, higher enzyme loading lead to faster initial reaction rate and higher amount of RDS was released as compared to lower enzyme loadings. Rosgaard *et al.* (2007) suggested that this might be due to higher conversion efficiency which was achieved as a result of high enzyme to substrate ratio. In this study, increasing enzyme concentration above 300 g/L lead to decline in RDS, which is contrary to the findings of Gama *et al.* (2017) where, increase of 0.4 to 0.6 mg enzyme/g substrate resulted in similar amount of RDS. The possible reason for difference in results might be the increased viscosity of the enzyme solution which lead to mass transfer limitations resulting in low conversion efficiency.

Duration of enzymatic pretreatment was varied from 30 to 120 min at 50ºC and at enzyme concentration of 300 g/L the RDS were rapidly released in first 30 min, followed by gradual increase till 120 min. Although, maximum RDS were obtained after 120 min in both EP-I and EP-II i.e. 0.260±0.021 g/g DM and 0.301±0.023 g/g DM respectively, 30 min of enzymatic pretreatment where, 0.183±0.020 g/g DM RDS were obtained in EP-II was selected as optimum parameter to reduce energy and enzyme input (Fig. 2C). To find the substrate concentration at which above optimized parameters would release maximum RDS, the amount of AP was varied from 1-5 g (Fig. 2D). With increase in substrate concentration the amount of RDS liberated also increased in a linear manner. In EP-I maximum RDS (0.377±0.026 g/g DM) were released at substrate concentration of 3 g while in EP-II, 4 g of substrate resulted in highest amount of RDS (0.431±0.007 g/g DM). In both the cases, with further increase in substrate concentration there was no significant increase in RDS which may due to saturation of enzyme at higher concentration of substrate. These findings are similar to the observations reported by Gama *et al.* (2017).

In all the experiments for optimization of pretreatment for apple pomace it was observed that combined pretreatment resulted in release of more RDS than enzyme or hydrothermal pretreatment alone.

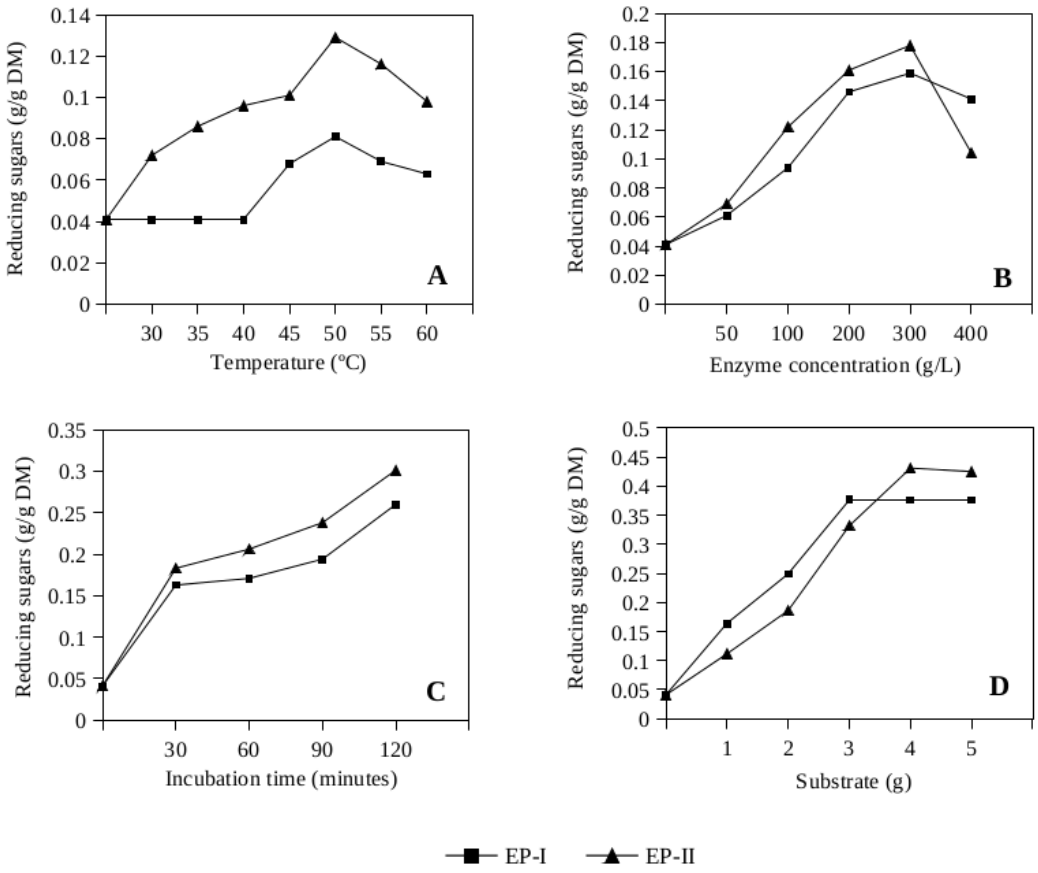

Fig. 2. Optimization of enzymatic pretreatment for reducing sugar release from apple pomace on the basis of (A) temperature (B) enzyme concentration (C) duration and (D) substrate concentration.

According to Xiao and Anderson (2013), the enzymatic digestibility of other cell wall components is obstructed by presence of pectin in the biomass. Use of pectinase enzyme prior to hydrothermal pretreatment resulted in removal of pectin from cellulose and hemicellulose network making it more susceptible to the action of hydrothermal pretreatment.The optimum conditions for enzyme pretreatment were; temperature, 50ºC; enzyme concentration, 300 g/L; treatment duration, 30 min and substrate concentration of 4 g combined with hydrothermal pretreatment at 121ºC for 30 min. Using the above optimized conditions 43.1 g of reducing sugars from 100 g of pretreated apple pomace was obtained.

C. Ethanolic fermentation using apple pomace as substrate

In this study, for the optimization of solid-state ethanolic fermentation, pretreated apple pomace was used as substrate and the effect of various parameters (relative initial moisture in substrate, temperature, *S. cerevisiae* inoculum size, co-culture inoculum size and nitrogen source supplementation) was determined. The results of this study are shown in Fig. 3.

Also, in this study, 70.89% (w/w) moisture present in fresh apple pomace was presumed to be 100% (w/w) relative moisture. When relative initial moisture in apple pomace powder was increased from 10 to 40% (w/w), ethanol concentration remained almost constant ranging between 8.636% to 8.817% (w/w). Ethanol concentration started increasing from 50% (w/w) relative moisture and reached maximum (11.673±0.041% w/w) at 100% (w/w) relative moisture (Fig. 3A).

The results showed similar trend with findings of Roukas (1994) where moisture level of 70% (w/w) was found to be the best for achieving highest ethanol concentration (160g/kg) from carob pods using solid-state fermentation. The possible explanation was that, high moisture level was necessary for optimal growth of microorganisms and also ethanol production (Swain *et al.,* 2013). Variation in temperature from 25-40ºC significantly affected ethanol production (Fig. 3B).

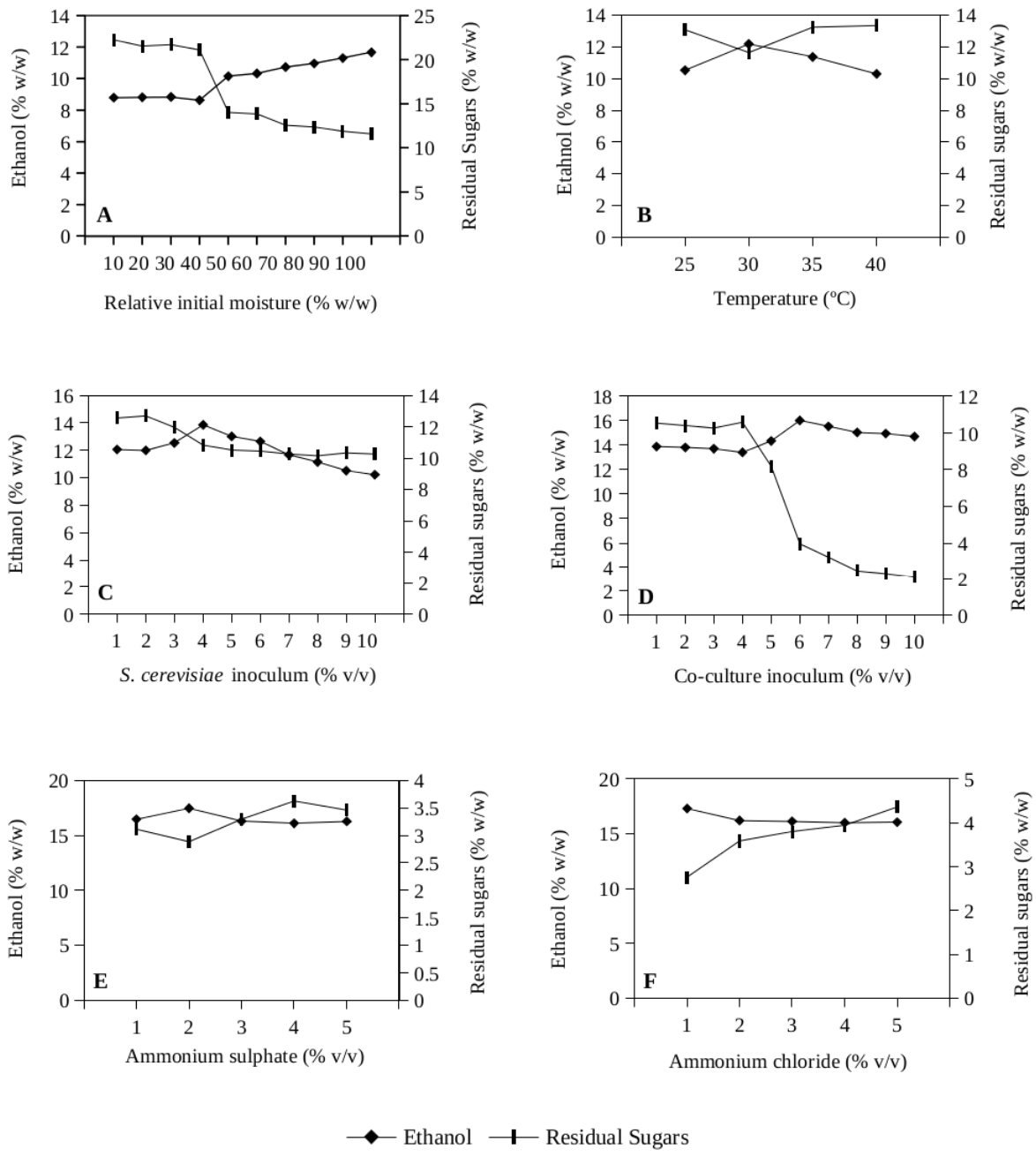

Fig. 3. Optimization of ethanol production from apple pomace using different variables; (A) relative initial moisture in AP (B) temperature of incubation (C) *S. cerevisiae* inoculum size (D) co-culture inoculum size (E) concentration of ammonium sulphate (F) concentration of ammonium chloride.

Temperatures above and below optimum temperature (30ºC) resulted in decreased ethanol concentration and at 30ºC ethanol concentration reached 12.172±0.020% (w/w). Temperatures higher than this lead to decreased ethanol production which may be due to decline in number of viable cells. Hang *et al.* (1986) and Roukas (1994) have reported temperatures between 25 and 30ºC to be optimum for ethanol production by *S. cerevisiae*.

The effect of inoculum size of *S. cerevisiae* on the production of ethanol was investigated by varying inoculum concentration from 1-10% (v/v) (Fig. 3C). Ethanol concentration gradually increased reaching its maximum (13.849±0.022% w/w) at inoculum size of 4% (v/v). With further increase in inoculum size i.e. from 5 to 10% (v/v) a significant decrease in ethanol production was observed while the amount of reducing sugars that remained unused was approximately 10% (w/w). The possible reason for this can be that unused sugars were composed of xylose, arabinose, rhamnose, uronic acids and unhydrolyzed polysaccharides that *S. cerevisiae* is not capable of fermenting. The reducing sugars that *S. cerevisiae* could ferment were consumed rapidly, probably for maintenance of cell mass which lead to decrease in ethanol production.

Zhang *et al.* (2011) used liquefied sweet potato mash (SPM) for simultaneous saccharification and fermentation where, glucoamylase (1.6 units/g SPM) and xylanase (1.5 units/g SPM) were added along with 3-15% (v/w) *S. cerevisiae* inoculum and showed maximum ethanol concentration of 112.4 g/L with 7% *S. cerevisiae* inoculum at 30ºC in 24 h. Kumar *et al.* (2014) reported 7.95% (v/v) ethanol from enzyme saccharified sweet potato flour by using 10% *S. cerevisiae* inoculum for 48 h fermentation at 35ºC.

The inoculum size of co-culture (APW-12) was increased from 1-10% (v/v) while keeping the inoculum of *S. cerevisiae* (primary culture) at 4% (v/v). The results of this study are shown in Fig. 3D which showed that, with the increase in inoculum size of co-culture from 1 to 4% (v/v), a slight decrease in ethanol production was observed, and this might be due to competition between the two types of microorganisms (*S. cerevisiae* and APW-12) for available resources. Ethanol production increased at 5% (v/v) inoculum and reached maximum i.e. 16.025±0.029% (w/w) at 6% (v/v) co-culture inoculum size. As the number of APW-12 cells increased in the production mixture they were able to hydrolyze cellulose and hemicellulose present in apple pomace by their extracellular enzymes which might have helped both *S. cerevisiae* and APW-12 to produce higher amount of ethanol. Any further increase in co-culture inoculum concentration resulted in decreased ethanol production as more reducing sugars were consumed, this can simply be attributed to increase in number of cells which were more inclined towards increasing their cell mass than towards producing ethanol. Baig and Dharmadhikari (2014) used co-culture of *S. cerevisiae* and *Pachysolen tannophilus* at inoculum concentration of 6% and 4% respectively, for ethanol production from enzymatic hydrolysate of cotton stalk and recovered 9.56 g/L ethanol. Itelima *et al.* (2013) used co-culture of *Aspergillus niger* and *S. cerevisiae* for conversion of pineapple, banana and plantain peels to ethanol. During simultaneous saccharification and fermentation process maximum ethanol yields of 8.34% (v/v), 7.45% (v/v) and 3.98% (v/v) were obtained with pineapple, banana and plantain peels respectively.

Fermentation medium was further supplemented with 1-5% (v/v) 1M ammonium sulphate and 1M ammonium chloride separately to determine the effect of nitrogen source on ethanol production. It can be seen in Fig. 3E that maximum ethanol (17.453±0.048% w/w) was produced when 1M ammonium sulphate concentration was maintained at 2% (v/v). Further increase in concentration of ammonium sulphate lead to decrease in ethanol production. Swain *et al.* (2013) used co-culture of *Trichoderma* sp. and *S. cerevisiae* for bioethanol production from sweet potato flour using solid-state fermentation. The fermentation was optimized at various parameters such as moisture content and initial pH of the substrate, temperature and nitrogen source. Four different nitrogen sources: urea, ammonium molybdate, ammonium sulphate and potassium nitrate were used in this study. Maximum ethanol production of 172 g/Kg substrate was obtained when 0.2% ammonium sulphate was used along with other optimized parameters i.e. 50 g substrate at pH 5.0, temperature 30ºC, inoculum size 10% (1:4 *Trichoderma* sp. : *S. cerevisiae*), moisture content of substrate 80% and incubation time of 72 h.

Addition of ammonium chloride in the present study at 1% (v/v) produced 17.295±0.048% (w/w) of ethanol, decreased ethanol production was observed at more than 1% ammonium chloride concentration (Fig. 3F). Peng (2018) investigated bioethanol production from Camellia seed meal using *S. cerevisiae* under optimized fermentation conditions (ratio of calcium and magnesium ion 1:1, yeast addition ratio 0.4% and ammonium chloride addition ratio 0.7%) and reported maximum ethanol yield of 95.4%.

In present study, the optimum conditions of solid-state fermentation for ethanol production were observed as; relative initial moisture in apple pomace, 100% w/w; temperature, 30ºC; inoculum size (*S. cerevisiae*), 4% v/v; co-culture inoculum size (APW-12), 6% v/v and nitrogen source concentration (1M ammonium sulphate), 2% v/v.

CONCLUSION

The present study reveals that combination of enzymatic and hydrothermal pretreatment increased reducing sugars availibility in apple pomace by 10.5 fold from initial level of reducing sugars. Also, this pretreatment process does not involve use of any chemicals and lasts only for one hour, making it simple and cost effective for use at industrial scale. Furthermore, optimization of solid-state fermentation for ethanol production by using pretreated apple pomace as substrate resulted in 17.5 g ethanol from 100 g of apple pomace.

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