Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Bioethanol production under multiple stress condition by a new acid and temperature tolerant *Saccharomyces cerevisiae* strain LC 269108 isolated from rotten fruits

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ARTICLE INFO

Keywords:
Dioscorea rotundata
Saccharomyces cerevisiae
Thermotolerance
Acid tolerance
Ethanol
Simultaneous saccharification and fermentation

ABSTRACT

Milled pulp flour from spoilt *Dioscorea rotundata* tubers was investigated as potential feedstock for bioethanol production using a new isolate of *Saccharomyces cerevisiae* strain LC 269108 displaying both thermotolerant and acid-tolerant properties. Fermentation was implemented by simultaneous saccharification and fermentation (SSF) for 60 h at pH 5.5 and temperatures of 30 °C and 40 °C. The results showed that the isolate metabolized a narrow range of carbon compounds, showed capacity to ferment many sugars (with gas evolution) and exhibited tolerance to acidification (up to 70 mM of acetic acid) under high temperature conditions. The time course of fermentation showed that the peak ethanol concentrations were 7.15 ± 0.08% at 30 °C and 7.29 ± 0.53% at 40 °C after 12 h and 48 h, respectively. In batches spiked with 50 mM acetic acid, the final ethanol concentration decreased to 6.30 ± 0.10% at 30 °C and to 5.50 ± 0.26% at 40 °C. No significant difference ($P > .05$) was found between the concentrations of ethanol produced at 30 °C and 40 °C. However, the ethanol concentration obtained from a culture containing 50 mM acetic acid was significantly lower ($P < .05$) than the value obtained in the control experiment (no acetic acid). This new isolate has a great potential for fermentation of acid-pretreated substrate at high temperature thereby reducing the cooling costs and the risk of microbial contamination.

1. Introduction

Ethanol provides energy that is sustainable and less carbon intensive than fossil oil. It reduces air pollution due to its cleaner emissions [1] and is an excellent raw material for many synthetic chemicals. One major problem confronting bioethanol production is the availability of raw materials. Fuel ethanol production from food crops raises deep food security concerns particularly in the developing countries due to increasing human population and dwindling land and food supplies. Bioethanol can be produced from various cellulosic feedstocks. Lignocellulosic biomass is the most promising feedstock due to its availability and low cost. It is the most abundant and sustainable resource and therefore the most attractive option for bioethanol production [2].

However, during production, bioconversion of cellulosic materials to fermentable sugars poses serious challenges because it is harder to depolymerize cellulosics than starch [3]. Presently, one of the most widely employed conversion methods is high temperature pretreatment with dilute acids [4–9] because it has been shown to increase cellulose digestibility of pretreated residues [6]. Often, after pretreatment, the final products must be neutralized by the addition of alkali to a pH consistent to the microbial requirement before fermentation of the sugars to ethanol. In large scale processes, the cost of chemicals can add significantly to the overall cost of production. Even during fermentation of starchy materials under non-strict sterile conditions, contamination by various strains of bacteria leads to acid production, and thus acidification of the culture broth. The isolation of yeasts capable of fermentation without neutralization will be highly beneficial.

Starch hydrolysis and acid pretreatment of lignocellulosics result in the release of several products that are inhibitory to microorganisms. The negative effects of furfural, 5-hydromethyl furfural (HMF), acetate, formate, hydroxybenzaldehyde (HBD), vanillin, and phenol compounds on yeast metabolism have been reported [9–11]. Therefore, the use of yeasts capable of overcoming the limitations imposed by the presence of these compounds during ethanol production is imperative as it will

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https://doi.org/10.1016/j.procbio.2018.01.016
Received 7 September 2017; Received in revised form 26 December 2017; Accepted 24 January 2018

increase the yield and eliminate the requirement for additional medium modification to suit the conditions of the fermenting organisms.

During fermentation, temperatures rise due to the activities of microorganisms [12] and must be controlled by a cooling mechanism. High temperature conditions have the tendency to jeopardize ethanol production and diminish final product concentrations. The prospects of conducting fermentation at high temperatures using thermotolerant yeasts is attractive and serves two major advantages of reducing the cooling costs and decreasing the risk of bacterial contamination [13].

Currently, it is estimated that more than 50% of the cost of microbial products are accounted for by the feedstock alone [14–16]. Therefore, increasing ethanol yield is crucial and obligatory to the sustainability of the bioethanol project. In the present study, spoilt yam was investigated as a potential feedstock in order to reduce the cost of production and increase the economic viability of the process. Nigeria is currently the world largest producer of yams accounting for over 76% of the global production, ahead of Cote d'Ivoire and Ghana. In 2011 alone, Nigeria, produced 33 million metric tons of yam from 4475,356 ha of land [17]. Presently, the volume of spoilt yam is estimated at 40% of the annual harvested volume [18] due to the difficulty in preserving (storing) them over extended periods. Decomposing yam heaps are usually disturbing, unsightly and constitute public health dangers because they provide potential breeding grounds for numerous insect and animal vectors.

The biotechnological conversion of agricultural wastes into useful products and biochemicals is a sustainable way of addressing the menace of environmental pollution because treatment and management costs are significantly reduced by this approach [11]. Although the use of peels from yam and other tuberous and non-tuberous crops have been studied as potential resources for bioethanol production [19–21], no report hitherto has documented the suitability of spoilt tubers. In this study, we evaluated the potential of spoilt yam as feedstock for ethanol production. We isolated and characterized a robust *Saccharomyces cerevisiae* LC 269108 strain from rotten fruits in Nigeria. The yeast showed capacity for multi-stress tolerance to agents such as acid and high temperature conditions. The new strain showed potential for high ethanol production under non-optimum conditions from spoilt yams.

2. Materials and methods

2.1. Sample collection and preparation of rotten yam pulp flour

Yam tubers weighing approximately 5 kg total weight were obtained from the waste dump at a yam market near Nsukka, Nigeria and taken to the laboratory for evaluation. Spoilage was confirmed if the tubers differed significantly from fresh tubers with respect to texture, odor, color and overall appearance. The samples used in the present study were softer than normal, had very unpleasant odor with clearly disseminated mould appearance on the external surfaces. A cross section revealed a brownish discoloration with progressive encroachment around the white area. The tubers were taken to the Crop Science Department of the University of Nigeria, Nsukka where they were taxonomically identified as *Discorea rotundata* Poir. Fresh *D. rotundata* tubers are usually cylindrical in shape, the skin is smooth and brown and the flesh is usually white and firm. The thin external epidermal covering of the sample was removed with a cutter and the tuber pulp sectioned into small chips to facilitate drying. The chips were oven dried (80 °C) for 48 h and then milled into powder in a Fritsch Pulverisette 14 (Made in Germany). The pulp powder was sieved using a Horizontally Rotating Sieve SKH-01(Fujiwara Scientific Company) having a 90 μm pore size. Coarse materials were re-milled until fine light brown flour was obtained. The pulp flour was stored at −30 °C until required for use. This pulp flour was used in all the experiments, except for proximate analysis where peel flour was analyzed for comparison. The pulp flour was stored at −30 °C until required for use.

2.2. Isolation of yeast strains

The fruits used for the yeast isolation were hand-picked from a waste dump at Nsukka, Nigeria. Putrefying fruits such as pineapple, paw–paw and oranges were collected in polythene sample packs and transported to the laboratory. The tip of sterile picks was dipped into the 'soup' emanating from the fleshy decomposing regions and inserted into each 14 ml round capped tubes containing 3 ml YPD 10% (1% yeast extract, 2% peptone and 10% glucose) supplemented with chloramphenicol before incubating for 18–24 h at 42 °C. Loopfuls from tubes which showed significant bubbling and gas evolution were spread inoculated on fresh agar plates and incubated again. The composition of the solid medium was the same as the isolation medium except that agar (2.0%) was added. The developed colonies were sub-cultured twice to enhance purity of the cultures. For the purpose of selecting appropriate strain for use in the study, a loopful of each of the isolated yeasts were inoculated into separate batches of YPD in screw capped falcon tubes and incubated at 40 °C for 24 h. Samples taken from each tube were evaluated for ethanol concentration. From the data obtained, the high ethanol producing strains were selected and used in the stress (high temperature and acetic acid) tolerant experiment. Except otherwise indicated, all the media components were of analytical grade and supplied by Wako Pure Chemicals Co, Japan.

2.3. Sugars assimilation tests

Approximately 100 μl of the diluted pre-culture was added to C medium (7 ml) of the API 20C Aux test kit. Subsequently, 200 μl of the mixture was inoculated into the different cupules (containing different sugars) accompanying the kit according to manufacturer's instructions. The plate was then incubated for up to 72 h at 30 °C. The reactions were read by comparing with the growth controls and the result was confirmed by referring to the Analytical Profile Index.

2.4. Sugar fermentation and gas production

Approximately 20% of each sugar (glucose, fructose, galactose, maltose and sucrose) and 10x nitrogen base (20% peptone and 10% yeast extract) were prepared in separate reagent bottles, sterilized and cooled. Subsequently, one portion of each sugar solution was mixed with one portion of nitrogen base and diluted with eight portions of Milli-Q water. About 3 ml of the resulting mixture was dispensed into 15 ml capped falcon tubes. Thereafter, sterile Durham tubes filled with Milli-Q water were lowered in inverted positions into the capped falcons by a pair of sterile forceps. Then, 100 μl of the diluted 24 h preculture was inoculated into each tube and incubated at 30 °C and 40 °C for 24 h. Fermentation was confirmed by the appearance of bubbles at the top of the broth. Gas production was taken to have occurred if water was displaced from the tip of the inverted tubes as a result of carbon dioxide evolution.

2.5. Determination of stress tolerance of selected yeast strains

Fresh YPD agar (2% D-glucose, 1% yeast extract, 2% peptone and 2% agar; pH 6.92) was cooled to 50 °C–70 °C after sterilization (Sanyo Sterilizer MOV-2125) at 121 °C for 15 min. Thereafter, acetic acid (Ac) solution (pH 4.76) was added to final concentrations of 60, 70 and 100 mM before the plates were poured and cooled. The final pH of the respective media was 4.74, 4.68 and 4.55, respectively. A 24 h preculture of each selected strain was diluted to 1.0 OD before 10-fold decimal dilutions of each strain was made and drop inoculated (up to 10^4) on YPD plate containing different concentrations of acetic acid. The inoculation was made in series for each strain in order of decreasing cell concentration. The plates were incubated at 30 °C and 40 °C for 24 h.