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Research Article

Consolidated Bioprocessing in Solid State Fermentation for the Production of Bioethanol from a Novel Mixture of Surgical Waste Cotton and Waste Cardboard

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Abstract

Consolidated bioprocessing to simultaneously produce cellulolytic enzymes, saccharify the biomass and ferment the released monosaccharides to ethanol is a much sought after technique in the vast area of biomass to ethanol conversion. In the present work, 100 g of a mixture of surgical waste cotton and waste card board (1:1) ratio were subjected to CBP in a solid state fermentation mode. Yeast was grown for 48 h as sheets on 4 large nutrient agar petri-dishes (10 cm diameter). The (10% w/v optimized) yeast sheets were laid on the bottom sieve of the SSF aerated tray reactor. Over the yeast sheets were spread 100 g of surgical waste cotton and waste card board mixture. 4 filter papers (15 cm diameter) inoculated and incubated with *T. harzanium* for 5 days were laid over the bed of the cellulosic mixture. After 12 days of incubation for CBP in a static humidity chamber at 30-32°C (optimized), around 11.25 ± 0.1 g/L ethanol with a percentage cellulose conversion of 11.04% was produced and 30 ± 0.02 g/L glucose was left unconsumed. The enzyme activities at the end of 12 days were FPases: 2.4 ± 0.09 IU/mL, CMCases: 16.8 ± 0.1 IU/mL, Xylanases: 936.15 ± 0.1 IU/mL and Beta-glucosidases: 3917.5 ± 0.09 IU/mL.

Keywords: Consolidated Bioprocessing; Cellulolytic enzymes; Solid State Fermentation; Bioethanol.

Introduction

The biorefinery concept of employing waste lignocellulosic biomass as a source for the second generation biofuel production is due to its easy availability, inexpensiveness, and the environment friendly green approach used for production. Lignocellulosic biomass comprises 30-40% cellulose, 20-40% hemicellulose, and 10-20% lignin by weight. The recalcitrance of lignin impedes efficient conversion of the encased cellulose to ethanol. The conventional process of the conversion of biomass to ethanol involves a pretreatment step to reduce the recalcitrance of lignin, production of enzymes that hydrolyze the cellulose to fermentable monosaccharides, saccharification of the pre-treated biomass followed by fermentation of the fermentable monosaccharides in the enzymatic saccharified hydrolysate. Bioethanol fuel blends are a vogue now in flex fuel vehicles and also as a stand-alone fuel in modified automobile engines [1, 2] Consolidated Bioprocessing (CBP), a process which integrates all the above mentioned individual stages in one process, is a novel approach which is in its nascent stages.

CBP is a single economical process that shows potential for a single pot process for the conversion of Lignocellulosic Biomass to ethanol. A single genetically engineered microbial strain or consortia of strains with close cultivable temperatures are used in the process of CBP [3]. The process produces allows the production of enzymes, saccharification of the biomass by the enzymes and fermentation of the enzymatic hydrolysate all in a single process. No external enzyme is required to be added [4, 5]. Genetic engineering to produce a cellulolytic yeast, metabolic engineering of the pathways of cellulolytic organisms to produce ethanol by fermentation, a consortia of cellulolytic and fermentative organisms, protoplast fusion of cellulolytic and fermentative microbes, enhancing the natural CBP potential of certain *Clostridium* strains are some of the widely used approaches for CBP of lignocellulosic biomass to produce ethanol [6]. In the present work, a CBP was performed by using *T. harzanium* and *S. cereviseae* in a single Solid state fermentation process to convert a mixture of surgical waste cotton and waste cardboard to ethanol..

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Materials and methods

Materials

The microbial strains were purchased from American Type Culture Collection, USA. The nutrient rich media Potato Dextrose Broth procured from Hi-Media Laboratories, Mumbai, India was used for the growth of fungi. Nutrient agar was also procured from Hi-Media Laboratories, Mumbai, India. They were prepared according to the manufacturer's instructions and autoclaved at 121°C for 20 min. Other reagents used below were procured from Hi-Media Laboratories, Mumbai, India.

Vogel's media

The composition of Vogel's media (g/L) is as follows: Tryptone (1 g/L), Tri-sodium citrate (2.5 g/L), Di-Potassium hydrogen phosphate (5 g/L), Ammonium nitrate (2 g/L), Magnesium sulphate heptahydrate (1.4 g/L), Calcium chloride dihydrate 0.1g/L, Tween 80 – 0.2 % (v/v). A Trace element solution containing Citric acid monohydrate 5 g/L, Zinc sulphate heptahydrate 5 g/L; ferrous ammonium sulphate 1 g/L, Copper sulphate 250 mg/L, Manganese sulphate 50 mg/L ; Boric acid 50 mg/L, Sodium molybdate 50 mg/L was prepared. 1 mL of trace elements per litre of the total media components was added [7]. The pH was set at 5.5 before autoclaving.

A novel substrate mixture

A cost efficient and novel cellulose substrate was chosen for the production of cellulases. Surgical absorbent and non-absorbent cotton, cotton gauze, are used aplenty by the hospitals and clinics on a day-to-day basis [8]. These are considered as biological wastes and are discarded by the hospitals after a customary practice of disinfecting them chemically or by steam sterilization methods [8]. Waste cottons from the non-infectious, non-pathological division were collected. Cottons are known for their high percentage of celluloses [9]. Packaging card boards which are high in cellulose content were also chosen [10]. Supermarkets discard a lot of packaging material everyday when they are damaged and fungal-infested making them unsuitable for recycling purposes. In an attempt to using the cheapest cellulose substrate for the production of cellulases, we collected these waste materials. This would also decrease the generation and accumulation of waste. The usage of waste material for fuel production would be another added advantage of this choice.

Preparation of the novel cellulosic substrate mixture

The disinfected surgical waste cotton was subjected to a stage of autoclaving prior to washing them using a mixture of disinfectants [8]. Proper laboratory personnel protective equipments were used for the purpose. Once the cotton was processed using the above approaches, it was dried in a hot air oven at 30 degree Celsius until it was completely dry. Cotton contains 90% cellulose [9]. The solid components of the biomedical wastes consists of bandages and linen (30–35%), plastics (7–10%), disposable syringes (0·3–0·5%), glass (3–5%) and other general wastes including food (40–45%). Waste packaging card boards were collected from the wastes generated by the e-commerce industry packaging [10]. Damaged waste packaging card boards were washed with water repeatedly to get rid of visible dirt. The card boards were shredded to uneven sizes in a cardboard shredder (0.5 cm average size). After shredding, it was finely powdered using a mixer grinder. The dried cotton was pulverized to a fine powder though some of the strands remained intact. The card board and cotton mixture were used in a 1:1 ratio for the Solid state and Submerged fermentation processes for the production of cellulases. Waste packaging card boards were collected from the wastes generated by the e-commerce industry packaging [10].

Preparation of primary inoculum

In the present work solid state fermentation was to be performed in a self-designed aerated tray reactor. Primary inoculum of *T. harzanium* and *S. cereviseae* were prepared. Whatmann No.1 filter papers (15 cm diameter) were used for the primary culture of *T. harzanium*. The Whatmann No.1 filter papers were sprayed with Vogel's minimal media components. It was inoculated with liquid culture of *T. harzanium* by spraying the inoculum on top of the moistened filter paper. The filter papers were placed within large autoclaved petri dishes to maintain aseptic conditions and were incubated at 28°C for 5 days until the entire filter paper was covered with a uniform growth of the fungus. *S. cereviseae* inoculated by spread plating was grown on

Nutrient Agar in large petri dishes (10 cm diameter) for 48 hours. The yeast spread as a blanket over the layer of the agar. These sheets of yeast were used as primary culture for the CBP.

Optimization of the consolidated bioprocessing

The process of optimization of the Consolidated Bio-processing was done for three inoculums concentrations (w/v %) of the yeast biomass and three times of incubation for the process. 10, 12 and 14 days were the days of incubation while 5, 10 and 15% (w/v) of *S. cereviseae* biomass were used to finalize the optimum ethanol yield. The conditions and methodology to carry out the solid state Consolidated Bio-processing are as mentioned in Section 2.5 Solid State Consolidated Bio-processing.

Solid state consolidated bioprocessing

Four large sheets of *Saccharomyces cereviseae* (10% w/v of the substrate) were laid over the bottom sieve layer of the solid state aerated tray reactor of dimensions lbh (35 cm x 30 cm x 15 cm). Over the bottom sieve layer of the reactor having yeast , 100g of the autoclaved surgical waste cotton and waste cardboard mixture mixed with Vogel's minimal media components (1:2 solid:liquid ratio) were spread. In the reactor components, a pH of around 5.5 was maintained. A moisture content of around 50-60% (v/w) was maintained in the cellulosic substrate mixture. The filter paper having *T. harzanium* growths were removed out of the petri dishes and aseptically transferred to the top of the cellulosic substrate as a blanket layer. The tray reactors were placed inside a humidity chamber to keep the humidity around 80% and a temperature of 28°C. A constant aeration of around 0.5 vvm was maintained in the systems. Regular observations were done visually to check for the drying of the fermentation contents. If the components dried up due to aeration, a sterile pipette was used to moisten the fermentation components using autoclaved Vogel's minimal media components periodically under aseptic conditions. At the end of 9 days, a dark mat of the fungal hyphae entirely covered the surface and the total substrate contents. An optimized time of 9 days was required for complete growth. The media was moistened with minimal media and pressed using a wooden presser so as to drive the liquid along with the hydrolyzed sugars to the bottom yeast layer and enable fermentation to occur. A sample of the hydrolysate was collected from the bottom sample collection port at the end of 9 days to assess the enzyme activity of the produced cellulases.

Extraction of enzymes and ethanol

After 12 days of incubation, 2 volumes (200 mL) of double distilled autoclaved water similar to the Koji volume were used to run through the buffer port in the top of the reactor. A harvest port in the bottom collected the water which flowed down. A metal wire gauze filter was used to filter out any residual substrate components. A step of centrifugation was performed to remove the debris from the extract. The supernatant contains the enzyme and ethanol [11]. The total volume of the water used was 200 mL. Around 190 mL of the buffer was recovered from the Koji. The enzyme activity for the produced cellulases was measured using the standard IUPAC DNSA [12] reducing sugar estimation method. Ethanol produced was estimated using a HPLC.

$$\%\ Saccharification = \left\{\frac{(Total\ Sugars\ (g) \times 0.9)}{Weight\ of\ alkali - pretreated\ rice\ straw\ (g)}\right\} \times 100 \quad (1)$$

0.9 - The factor is used to convert polysaccharides to monosaccharides accounting for water uptake during hydrolysis.

% yield of saccharification = (Practical yield / theoretical yield) x 100 (2)

HPLC Analysis

A HPLC analysis was performed using an Agilent 1290 Infinity HPLC with a Refractive Index detector. The mobile phase was sulfuric acid 0.005 M. 20 microlitre of the samples were injected. Standards with known concentrations were run prior to analysis of unknown sample mixtures. The oven temperature was 60 degree Celsius and the detector temperature was 55 degree Celsius. The column was Agilent Hi-Plex H 7.7 mm x 300 mm x 8 mm.

Estimation of saccharification and cellulose conversion efficiencies

The efficiency of cellulose conversion was estimated as percentage cellulose conversion. The formula used to perform the quantification is given in eq. (3).

$$\% \text{ cellulose conversion} = \left\{\frac{(Ethanol)_f - (Ethanol)_i}{0.51 \times (f \times biomass) \times 1.111}\right\} \times 100 \quad (3)$$

$(Ethanol)_f$ – Final concentration of ethanol after fermentation (g/L), $(Ethanol)_I$ – Ethanol initial concentration in the broth (g/L), Biomass – dry biomass (g/L) at the beginning of fermentation, f – Fraction of dry biomass containing cellulose (g/g), 0.51-Conversion factor for the conversion of glucose to ethanol based on stoichiometric biochemistry of yeast and 1.111 – Converts cellulose to glucose equivalent.

Results and discussions

At the end of the CBP after 12 days, the enzyme activities were observed. The second column represents the Enzyme activity in IU/mL, the third column represents the enzyme activity per unit gram of dry solids in IU/g.ds and the fourth column represents the specific activity of the enzymes expressed in IU/mg (Table 1).

Table 1. enzyme activities after 12 days CBP

Enzyme	IU/mL	IU/g.ds	IU/mg
1. Fpase	2.405 ± 0.09	0.72	1.246
2. CMCase	16.832 ± 0.1	5.04	8.772
3. Xylanase	936.15 ± 0.1	280.8	485.05
4. Beta glucosidase	3917.5 ± 0.09	1175.2	2029.7

Optimization of consolidated bioprocessing

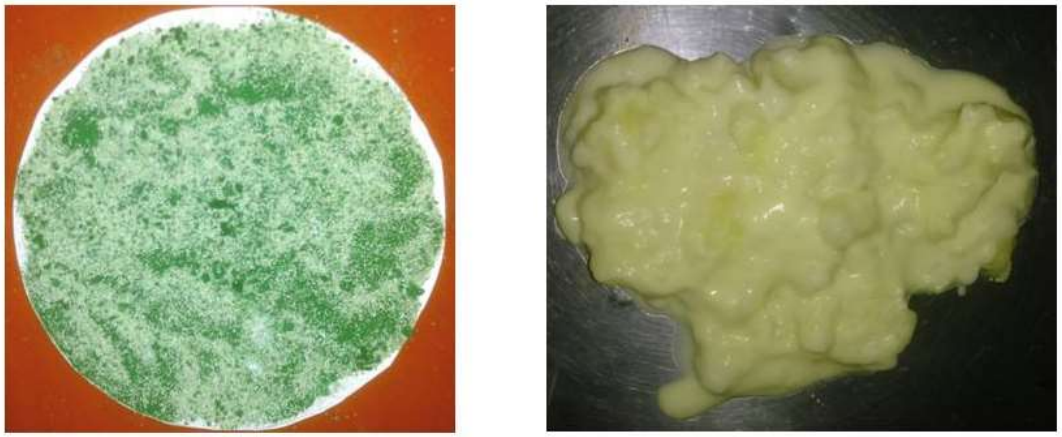
The process of CBP was optimized at various days of incubations: 10, 12 and 14 days and various % (w/v) of yeast biomass: 5%, 10% and 15% (w/v). The enzyme activities were measured at the end of 9 days and 12 days. The values of enzyme activities were the same as mentioned in Table 1 and did not increase with time. The total protein content of the fermentation was measured using Lowry's Assay and it was 1.93 ± 0.1 mg/mL. When 5% (w/v), 10% (w/v) yeast biomass and 10 days of incubation was carried out no ethanol was produced. However sufficient enzymes with activities mentioned above were present in the sample analyzed. In the next condition of 5%(w/v) yeast and 12 and 14 days of incubation around 2 ± 0.01 g/L ethanol was produced. This showed that higher days of incubation were required as the monosaccharides that were obtained through hydrolysis needed 2-4 days more to be fermented to ethanol.

When 10% (w/v) yeast biomass was used and 12 and 14 days of incubations were carried out, 11.25 ± 0.1 g/L of ethanol was produced which was the maximum. 11.25 ± 0.1 g/L ethanol was produced when 15% (w/v) yeast and 12 & 14 days incubations were carried out. When 15% (w/v) yeast biomass was used and 10 days of incubation were done, around 5 ± 0.01 g/L ethanol was produced which may be due to the further time required to ferment the monosaccharides to ethanol. After analyzing the amount of ethanol produced in the various conditions of CBP, 12 days of incubation and 10% (w/v) yeast biomass was fixed as the optimum condition to perform a CBP.

Consolidated bioprocessing in solid state fermentation

The optimum CBP condition of 12 days of incubation and 10% (w/v) yeast biomass was used to process 100 g of the (1:1) ratio of surgical waste cotton and waste card board mixture. The sample collected after CBP from the bottom showed around 11.25 ± 0.1 g/L Ethanol, 30 ± 0.02 g/L glucose and 5 ± 0.01 g/L xylose. 30 ± 0.02 g/L was the unconsumed glucose from the biomass. The solid state fermentation proceeded with the filter paper harbouring *T. harzanium* laid over the moistened cellulosic substrate bed [1, 8]. After 2 days of growth on the filter paper, the mycelia started penetrating the cellulosic substrate bed, two reasons could be attributed for the mycelia of *T. harzanium* penetrating the layer beneath the filter paper (Fig. 1). One is that the cellulose content of the filter paper got used up and the fungus had to go in search of newer cellulose for growth. The moisture in the cellulosic bed might have wetted the base of the filter paper making it further moist letting the fungus continue its

growth on the cellulosic bed after the filter paper was consumed.



(a) (b)

Fig 1. (a) *T. harzanium* grown on Whatmann No.1 Filter paper. (b) *S. cereviseae* biomass to be spread on the bottom sieve of the aerated tray reactor

As and when the Koji dried up, Vogel's media was used to moisten the substrate. The hyphae completely penetrated through the solid bed at the end of 4-6 days. Incubation was done at 30°C. Aeration was maintained at 0.5 vvm using internal aeration lines into the solid state bed. Too much aeration would cause drying up of the Koji while too little aeration would cause no growth over the solid state components. At the end of 9 days (optimized for Solid state fermentation of the cellulosic substrate), around 200 mL of autoclaved distilled water was run through the Koji components to enable the produced monosaccharides to reach the yeast layer. A wooden presser was used to maximize the amount of water with sugars reaching the yeast biomass bed. 9 days was the optimized time for a Solid state fermentation to complete. 48 hours was required for the sugar fermentation process using yeast to complete as known from previous experiments [13]. Saccharification may occur simultaneously as the enzymes are secreted from the fungal hyphae which were again known through previous experiments where after 48 hours of cellulase production, background sugars from the enzyme-hydrolyzed substrate start accumulating causing catabolite repression [14, 15, 16]. Though batch saccharification of cellulosic substrates using cellulases occurs at a higher temperature of 50°C for 48 h a considerably large amount of saccharification occurs when the enzymes work at a temperature of 30°C as well which is evident in this work and also similar works. In total 12 days were required for the process of CBP to complete.

The yield of ethanol was around 2.25 ± 0.01 g from 100 g of the cellulosic mixture showing a % cellulose conversion of 11.04%. A 100 g mixture of surgical waste cotton and waste card board would yield around 34-35 g of cellulose. In a normal batch saccharification process at elevated temperatures better saccharification yields would be obtained. When 30-35 g sugars were to be fermented by yeast [13] an ethanol yield of more than 15-20 g should have been obtained keeping in mind that the fermentation capability of *S. cereviseae* RW 143 which is 0.4 g ethanol/g glucose as estimated from previous experiments. But the yield is low here which could be because a lower concentration of sugar for a higher volume of yeast would result in more biomass formation rather than more ethanol fermentation. In an attempt to increase the saccharification efficiency after the SSF of the cellulosic substrate was complete and before the sugars could reach the bottom yeast layer, the temperature of incubation was raised to 50 degree Celsius. However, the yeast population did not survive the temperature [17]. Hence it was decided to maintain the temperature around 28-30°C and permit growth and fermentation. The amount of ethanol obtained using the above mentioned method was around 11.25 ± 0.1 g/L (Fig. 2). Similar studies carried out by researchers using various strains yielded ethanol quantities which are closer to the values obtained in this study, followed a Solid State Fermentation CBP approach using a single wild strain *F. velutipes* and mechanically pre-treated rice straw biomass which yielded 18.2 g/L

ethanol at the end of 15 days [18] used an anaerobic strain *Clostridium sp.* DBT-IOC-C19 in a submerged CBP process that yielded 0.7 g/L ethanol. In a novel SSF CBP approach followed by Simone et al, 2014 [19] pre-treated biomass was subjected to the action of a microbial consortia which yielded 19.4 g/L ethanol at the end of 7 days.

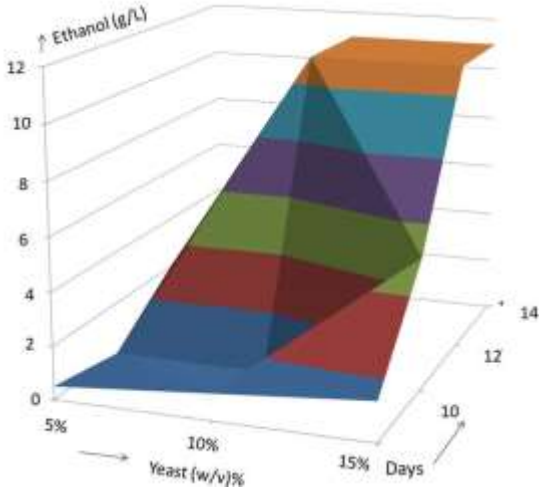


Fig 2. Optimization of the process of CBP at various times of incubation and various yeast concentrations

Though the yield shown in our study is comparatively lesser than the contemporaries, the salient features are stated below. No pre-treatment of the biomass was followed in the study. Expensive anaerobic culturing equipments were not used for the cultivation of the microbes during CBP. No expensive synthetic microbial biofilms were employed in the study. The substrate is from the waste generated from hospitals and supermarkets. In comparison to the CBP methods followed by contemporaries, the methodology proposed in the study is straight forward and easily adaptable.

HPLC chromatogram (Fig. 3) of the optimized Solid State Consolidated Bio-Processing showing the yield of 11.25 ± 0.1g/L Ethanol at 36.45 min retention time. Unconsumed 30 ± 0.02 g/L glucose was observed at a retention time of 9.8 min and 5 ± 0.01g/L xylose was seen at a retention time of 10.4 min.

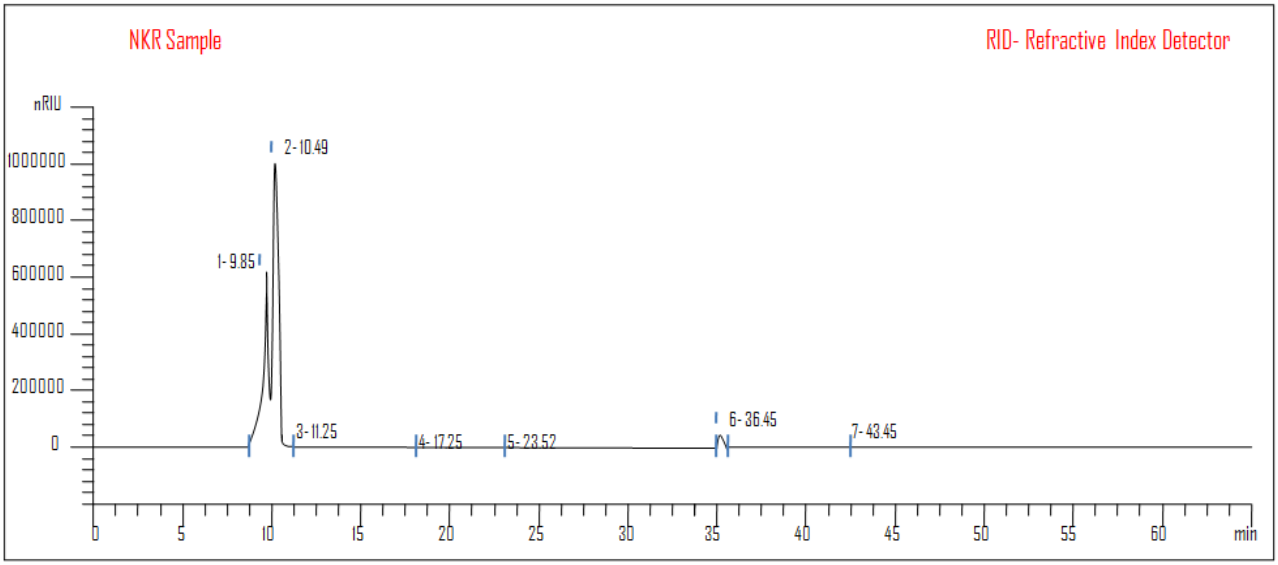


Fig. 3. HPLC chromatogram bioethanol obtained by consolidated bioprocessing

Conclusions

CBP involves a consolidated process of converting biomass to ethanol by saccharifying the biomass and fermenting it into ethanol, all in a single process. A process of optimization was carried out prior to the commencement of the actual process and estimation of yields. In a tray reactor and in a solid state fermentation mode, *S. cereviseae* biomass and *T. harzanium* were simultaneously cultivated using surgical waste cotton and waste card board as the substrates to yield ethanol. The yield of ethanol is slightly lower than that obtained using individual process of enzyme production, saccharification and fermentation. However, the process is much economical than the conventional multi stage process of ethanol production. The time required for the completion of the process is 12 days which is much lesser when compared to the other lengthier conventional processes which take several days to reach completion.

Conflicts of interest

The authors declare no conflict of interest.

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