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Single pot biovalorization of food waste to ethanol by *Geobacillus* and *Thermoanaerobacter* spp.

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ABSTRACT

The current study was focused on developing a green thermophilic bioprocess to produce bioethanol from the food waste. The stored energy in food waste can be used for bioethanol production that can also help in reducing the land and environment impact of current food waste management processes. *Geobacillus thermoglucosidasius* was used to produce ethanol from food waste in a single pot at 60 °C. Scaling up the single pot bioprocess from 165ml serum bottles (165ml) to 1L bioreactor increased the ethanol yield from 3.03 g/L to 13 g/L. With scaling up to 1L reactor improved the substrate mass recovery from 39.2% to 92.8%. The ethanol production in 1L was further increased to 16.1g/L by the sequential cultivation of *Geobacillus thermoglucosidasius* and *Thermoanaerobacter ethanolicus*. Further, scaling up to 40L reactor gave an ethanol yield of 18.4 g/L and improved the ethanol productivity from 0.07 g/L/h (1L reactor) to 0.15 g/L/h (40L reactor). The sequential cultivation of thermophiles was able to produce ethanol from food waste without pretreatment, giving 70.1L of gasoline equivalent per ton of dry food waste. This is the first report of sequential cultivation of thermophiles for ethanol production using food waste and scaling up to pilot plant.

1. Introduction

Unplanned buying, and discarding material, lack of suitable infrastructure, and processing and packaging facilities etc. result in 1.3 billion tons/year of food waste globally [1]. Such enormous quantity of food waste not only represents a direct capital loss, but also represents a waste of land, water, energy and inputs resources used in the production, and waste management [2]. Food waste, a rich biomass harboring 35.5–69% carbohydrates, 3.9–21.9% proteins, oils and fats, and organic acids can be a very good source for bioethanol production [3]. The use of bioethanol is promising when compared to other biofuels due to its ease of blending with gasoline to use in the current automotive set up, latent heat of vaporization 839.7 kJ/kg [4]; octane booster [5], and lower toxic emissions [6]. The use of corn, sugarcane, and other food based substrates for ethanol production is discouraged because of the food vs fuel competition and related environmental, economic and agricultural issues [7]. Hence, new economical and abundantly available substrates are constantly searched for bioethanol production and food waste is a sustainable, economic and ubiquitously available bioenergy substrate.

Several studies reported the use of food waste for ethanol production by various ethanologenic microorganisms. [8]; reported 45 g/L of ethanol production using kitchen waste by *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia stipitis* [9]; reported 46.9 g/L of ethanol by *Paenibacillus chitinolyticus* strain CKS1 and *S. cerevisiae* using damaged rice grains, and [10]; reported ethanol production (191 g/kg substrate) from organic fraction of municipal solid waste by *Mucor indicus* strain CCUG 22424. Most of reported studies use a pretreatment step to produce fermentable sugars from the food waste in spite the fact that pretreatment is considered one of the most energy and cost intensive step in bioethanol production

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process [11]. Mahmoodi and coworkers (2018a) used dilute H_2SO_4 pretreatment followed by treatment with Cellic Ctec 2 to pretreat the organic fraction of municipal solid waste. The sequential pretreatment of FW with HCl (1.5% v/v at 90 °C for 3 h), and enzyme (glucoamylase −85U/ml at 100 rpm for 6 h) gave a sugar yield of 103.4 g/L and 10.92 g/L of ethanol [12]. In another study Ghazali and coworkers [13] also increased the ethanol production from 4.4 g/L to 16.4 g/L after the pretreatment of the durian seeds with dilute acid (0.113M H_2SO_4) prior to enzymatic hydrolysis. Furthermore, most of the studies use mesophilic microorganisms for fermentation. Matsakas and Christakapolus produced 6 g/L of using untreated household food waste with *Saccharomyces cerevisiae* [14]. There are very few studies on ethanol production with food waste using thermophiles without pretreatment [15]. The thermophilic microorganisms offer several advantages over the mesophilic counterparts due to their wide range of substrate utilization, microbial contamination control, thermostable hydrolytic enzyme production, and higher kinetic rates [16]. Hence, the use of thermophiles will be advantageous for industrial in bioprocesses.

Here, we report ethanol production from food waste obtained from a local university cafeteria using a thermophilic facultative anaerobe *G. thermoglucosidasius*. The ethanol production was further improved by sequential cultivation of *G. thermoglucosidasius* and *T. ethanolicus* and scaling up. This is the first report of sequential cultivation of thermophiles for ethanol production using food waste without pretreatment and scaling up to pilot scale.

2. Materials and methods

2.1. Microorganism's cultivation and maintenance

G. thermoglucosidasius (ATCC 43742) and *T. ethanolicus* (ATCC 31938) were procured from ATCC and were grown and maintained on the media as suggested by ATCC.

2.2. Ethanol production-

2.2.1. In serum bottles-

For ethanol production simple sugars (glucose and xylose) and starch were used at a concentration of 2% and 10% (w/v), respectively, in 100 ml of the USYE media, used previously for ethanol production with *G. thermoglucosidasius* [17]. 100 ml of the media was added to the 165 ml serum bottles with pH adjusted to 6.75 using 6M NaOH. The serum bottles were sealed and crimped by butyl rubber septa and aluminum caps, respectively, and autoclaved at 121 °C and 15 psi for 15 min. Filter-sterilized biotin (12.5 mM), urea (20 mM), trace mineral salt solution and sugars were added to the media after autoclaving. 5% (v/v) of actively growing cells of *G. thermoglucosidasius* were added to the serum bottles that were incubated at 60 °C and 200 rpm. 1.5 ml of the samples was collected at predetermined time intervals, centrifuged, and the supernatant was kept at −20 °C until further analysis. 10% (w/v) (on dry basis) of blended food waste was used in 300 ml of USYE media in 500 mL serum bottles prepared as explained above. The food waste (Table 1) was collected from a local cafeteria at South Dakota School of Mines and Technology, Rapid City, South Dakota, USA (44.0742° N, 103.2059° W) and was kept at 4 °C until used for fermentation. The ethanol yield '$Y_{P/S}$' (g/g) and ethanol productivity 'q_p' (g/L/h) were measured using the equation below:

$$Y_{P/S}\ (g/g) = P_E\ (g) \Big/ S\ (g) \quad (1)$$

Table 1
Characteristics of the food waste used in the experiment on the dry weight basis. The values are mean of triplicate measurements with SD ± 3.

Parameters	Value
pH	5.56 ± 0.6
Moisture (w/w %)	71.6 ± 1.86
Total solid (TS) (w/w %)	29.3 ± 2.45
Total volatile solid (w/w %)	12.3 ± 1.23
Fermentable nitrogen (w/w %)	0.73 ± 0.18
Ash (w/w %)	6.34 ± 0.92
Total sugars (w/w %)	56.13 ± 3.56
Starch sugars (w/w %)	34.5 ± 1.03
Proteins (w/w %)	8.75 ± 0.82
Lipids (w/w %)	6.57 ± 0.72
Cellulose (w/w %)	18.1 ± 2.7
Hemicellulose (w/w %)	3.53 ± 0.22

$$q_P\ (g/L/h) = P_E\ (g) / [\text{Volume (L)*Time (hours)}] \quad (2)$$

where P_E= Amount of ethanol produced.

S =Amount of substrate utilized

2.2.2. Bioreactor

1Liter (L) DASGIP and 40 L BioFlo 510 reactor (Eppendorf, Hartford, CT, USA) were used for ethanol production with 10% and 20% (w/v) of the blended food waste, respectively. 1L DASGIP reactor with USYE medium, added to 50% of the total volume of the bioreactors, was autoclaved at 121 °C for 15 min at 15 psi. In case of 40L reactor the media was sterilized in place at 121 °C for 15 min at 15 psi. After autoclaving the pH probe was calibrated using a 2-point calibration method in both the bioreactors. The dissolved oxygen (DO) probe was calibrated with oxygen saturated conditions as 100% and nitrogen saturated conditions as 0%. The pumps were calibrated for adding acid (0.1N H_2SO_4) or base (10N NaOH) at 15 mL/min in an event of pH change. 5% (v/v) of freshly grown cells of *G. thermoglucosidasius* were added to the bioreactor. The reaction mixture was agitated at 300 rpm for 12 h and after that the agitation was reduced to 100 rpm. With the reduction in the rotational speed, the supply of air was also reduced from 1 vvm (volume of air to volume of media) to 0.2 vvm after 12 h.

2.3. Sequential co-culture of thermophilic microbes

G. thermoglucosidasius, and *T. ethanolicus* were sequentially cultivated to improve the ethanol production using food waste. 5% (v/v) of the actively growing culture of *G. thermoglucosidasius* was added to the bioreactor at 0 h. The reaction mixture was agitated at 300 rpm for 12 h and after that the agitation was reduced to 100 rpm. Along with the reduction of rotational speed the supply of air was also reduced to 0.2 vvm from 1 vvm after 12 h. The air supply to the bioreactor was stopped at 90 h. After 96 h, 10% (w/v) of *T. ethanolicus* cells grown for 15 h was added to the bioreactor during sequential cultivation. To improve the ethanol production in 1L bioreactor, three different changes were made- (a) *T. ethanolicus* cells were added at 48 h interval after t = 96 h, (b) *T. ethanolicus* cells and 20 ml (5X) minimal media were added at 48 h interval after t = 96 h. The composition of the minimal media per liter was 0.1 g nitrilotriacetic acid, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g NH_4Cl, 0, 1.8 g of 85% H_3PO_4, 1 ml of Nitsch's Trace element solution, and pH- 6.75 (using 6M NaOH). And (c) *G. thermoglucosidasius* cells were added at 48 h interval from t = 0 h to t = 96 h. In case of 40L bioreactor, *G. thermoglucosidasius* cells (4 g/L) were added at t = 12 and 36 h.

Similarly, the *T. ethanolicus* cells (6 g/L) were added at t = 48, 72, 96 and 120 h along with 50 ml of 20X media. No media was added with the addition of *G. thermoglucosidasius* cells. Samples were withdrawn periodically, centrifuged to remove cells and residual solids, and kept at −20 °C prior to HPLC analysis.

2.4. Analytical methods

The fermentation metabolites were detected by HPLC (Shimadzu LC20; Columbia, MD, USA) equipped with a 300 × 7.8 mm Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). One ml of the samples was centrifuged at 10,000 rpm for 10 min. The supernatant was removed, filtered using 0.2 μm pore size membrane filters (Gelman Acrodisc), injected onto a heated column (50 °C) and eluted at 0.45 ml/min using 5 mM H_2SO_4 as the mobile phase. The composition of gas was measured using a gas chromatograph (Agilent 7890A; Santa Clara, CA, USA), which had a thermal conductivity detector (TCD) and a Porapak Q column (Aw, 80–100 mesh, 6m × 1″ × 18″). 100 μL of the sample was periodically withdrawn from the serum bottles using a gas tight syringe (Hamilton Company, Reno, NV) and injected to the sampling port in GC. The carrier gas (N_2) was kept at a flow rate of 10 ml/min and the temperatures for injector and detector were 70 and 100 °C, respectively. The oven was operated at the starting temperature of 35 °C for 1 min, followed by a 5 °C/min ramp to reach 50 °C and final hold for 2 min at 50 °C (total run time 4.5 min).

2.5. Enzyme activities

2.5.1. Amylase activity

The amylase activity was determined using dinitrosalicylic acid (DNS) assay [18]. Equal volumes (0.5 ml) of enzyme and starch solution (0.2% w/v) in phosphate buffer (pH 7.0, and 0.1 M) were added to a test tube and incubated at 60 °C for 10 min. The reaction was stopped by adding 1.5 ml of DNS to the test tube and again incubated in a boiling water bath for 10 min. After incubation the test tubes were cooled on the ice for color stabilization and absorbance was measured at 540 nm. The concentration of maltose was measured against standard curve for maltose One unit (U) of the amylase was defined as the rate of production of 1 μM of reducing sugar (as maltose) from 0.2% soluble starch in 1 min at 60 °C and pH 7.0.

2.5.2. Glucoamylase activity

The glucoamylase activity was determined by end point analysis method [19]. Equal volumes (1 ml) of enzyme and starch solution (0.2% w/v) in phosphate buffer (pH 7.0, and 0.1 M) were added to a test tube and incubated at 60 °C for 10 min. The reaction was stopped by boiling the solution for 10 min 10 μL of the solution mixture were transferred to a fresh test tube and 1 ml of the GOD-POD reagent (Stanbio, Boerne, TX, U.S.A.) was added to the test tube. The tubes were incubated at 37 °C for 5 min. After incubation, the test tubes were cooled on the ice for color stabilization and absorbance was measured at 500 nm. The concentration of glucose was measured against standard curve for glucose. One unit (U) of the glucoamylase was defined as the rate of production of 1 μM of glucose from 0.2% soluble starch as a substrate in 1 min at 60 °C and pH 7.0.

2.6. Material balance and gasoline equivalence

The material balance was carried out in terms of mass balance for food waste. The energy efficiency for conversion of food waste to ethanol was calculated using the following equation:

$$\eta = xEthanol*EEthanol*100/\Delta H_{FW}*M_{FW} \quad (3)$$

where η represent energy efficiency, xEthanol represents the amount of ethanol produced in moles, EEthanol represents the energy density of ethanol (26.8 MJ/kg), ΔH_{FW} represents the heat of combustion for food waste, and M_{FW} represents the amount of food waste used. The ethanol equivalent to gasoline was calculated as the following equation:

$$\text{Energy in 1.5 gallon of ethanol} = \text{Energy in 1 gallon of gasoline} \quad (4)$$

3. Results and discussion

3.1. Growth and ethanol production with simple sugars

When grown on glucose and xylose, *G. thermoglucosidasius* utilized both the sugars for ethanol production. With glucose and xylose, a maximum ethanol production of 1.84 g/L equivalent to 0.04 mol/L, and 1.18 g/L equivalent to 0.026 mol/L, respectively, was obtained. The ethanol produced with glucose corresponded to a yield of 0.75 mol ethanol/mol glucose or 0.19 g ethanol/g glucose [37.5% of the maximum ethanol yield (1.00 $C_6H_{12}O_6$ → 2.00 C_2H_5OH + 2.00 CO_2 or 0.51 g ethanol/g glucose)], after 71 h (Fig. 1A). With xylose as the carbon source, the ethanol yield was 0.126 mol ethanol/mol xylose or 0.043 g ethanol/g xylose [7.6% of the theoretical maximum (1.00 $C_5H_{12}O_6$ → 1.66 C_2H_5OH + 1.66 CO_2; 1.67 mol ethanol/mol xylose or 0.51 g ethanol/g xylose)]

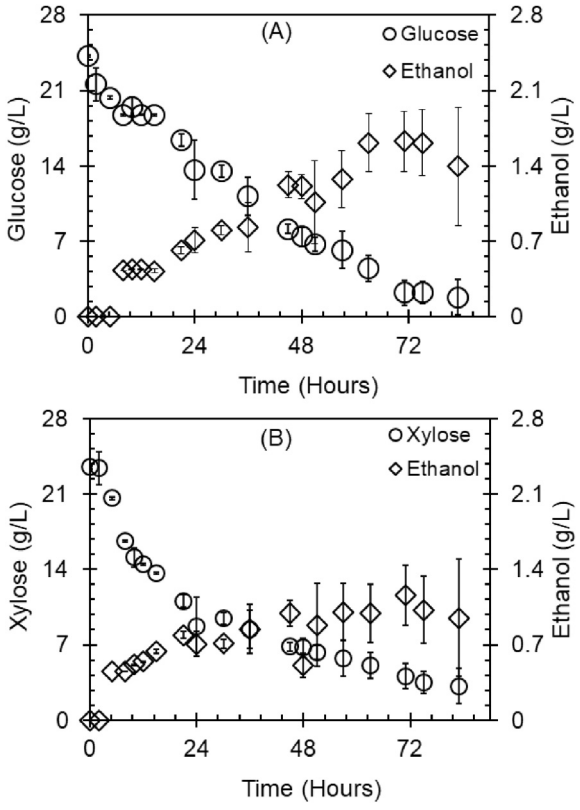

Fig. 1. Ethanol production and sugar consumption profiles with (A) glucose and (B) xylose by *G. thermoglucosidasius*. The values are mean of triplicates with standard deviation ± 3.

(Fig. 1B). Glucose was the preferred sugar by *G. thermoglucosidasius* as >98% of the glucose in the media was consumed, whereas only 80% of the initial xylose got consumed.

Cripps and coworkers (2009) reported a yield of 0.10 g ethanol/g glucose by *G. thermoglucosidasius* with 3% (w/v) of glucose. In addition to ethanol lactic acid, formic acid and acetic acid were the main soluble microbial products (SMPs) with both the sugars (Table 2). Cripps and coworkers (2009) also reported lactic acid, formic acid, and acetic acid by *G. thermoglucosidasius* during fermentation with glucose [17]. Lactic acid and acetic acid had been observed as the main SMPs during fermentation with other thermophilic bacteria also. *Clostridium thermocellum* also produced ethanol, acetic acid and lactic acid as the main SMPs [21]. Similarly, *Caldicellulosiruptor* and *Thermoanaerobacterium,* when grown with 25 mM glucose at 72 °C, also produced acetate and lactate as the fermentation products in addition to ethanol [22].

With glucose and xylose, respectively, 95.7 and 69.8% of the utilized carbon by the organism was recovered in the form of cell biomass, and metabolites produced (Table 2). The difference from the maximum (100%) can be attributed to the unquantified metabolites produced during the production. The difference in the amount of carbon recovered among the two sugars can be attributed to the different uptake mechanisms of the two sugars. Fig. 1A and B also showed that glucose utilization was faster than xylose. It had been shown previously that glucose and xylose are transported into the cells by different mechanisms. Where xylose transfer is mediated by a D-xylose binding protein in a facilitated diffusive manner the glucose transport occurs by ion exchange mediated active phosphorylation of glucose, which is faster as compared to the facilitated diffusion, resulting faster uptake, assimilation and hence ethanol production [23].

3.2. Ethanol production with starch

For ethanol production with starch, *G. thermoglucosidasius* was grown on 10% w/v starch at 60 °C and produced 3.6 g/L of ethanol in 48 h (Fig. 2A). With starch, acetic acid (0.94 g//L) and lactic acid (1.52 g/L) were also produced. Interestingly, further incubation beyond 48 h did not increase the ethanol production as was observed with glucose.

The starch hydrolysis requires a concerted action of amylase and amyloglucosidase, respectively, to convert it into maltooligosaccharides and further to glucose [24,25]. With *G. thermoglucosidasius* maximum activities for α-amylase and amyloglucosidase, respectively, observed were 4.65 U/ml and 10.1U/ml after 96 h (Fig. 2). Higher amyloglucosidase activity compared to amylase can be the plausible reason for higher glucose content in the reaction system. Higher glucose content possibly resulted in osmotic stress and hence, no increase in ethanol content after 48 h.

HPLC analysis showed a significant concentration of glucose was present after 48 h of fermentation (Table 2). No glucose was observed in the fermentation broth till 48 h. This suggested that the starch hydrolysis and glucose utilization occurred simultaneously till 48 h but started accumulating in the fermentation broth afterwards. Similar results were obtained with *Schefferomyces shehate* grown on starch that also hydrolyzed starch and fermented glucose simultaneously with no free glucose observed in the fermentation broth [26]. It produced 9.2 g/L of ethanol when incubated with 10% (w/v) starch at 25 °C after 10 days and showed increased amylase and amyloglucosidase activities. Thus, the enzymatic activities had a significant effect on the ethanol production, and it can be concluded that osmotic stress exerted by high concentration of glucose caused inhibition of metabolic activity in *G. thermoglucosidasius*.

More ethanol production (3.68 g/L) was observed with 10% (w/v) of starch when compared to 10% (w/v) of glucose (1.84 g/L). This is because glucose is a readily utilized source, whereas starch is a complex polysaccharide that required enzymatic hydrolysis to release glucose. A higher glucose concentration will exert more osmotic stress than a similar concentration of starch. The osmotic stress induced by higher glucose concentration can interfere with the physiological functions of the microorganism resulting in lower ethanol production. Hence, a higher ethanol production was observed at higher concentration of starch compared to glucose.

3.3. Ethanol production with food waste

3.3.1. In serum bottles

With 10% (w/v) blended food waste as substrate an ethanol yield of 3.03 g/L was obtained after 96 h (Fig. 3A). The ethanol yield with food waste was less than that obtained with starch (3.68 g/L). It can be attributed to the fact that the food waste is a more complex substrate as compared to the starch [27]. The fermentative product profile showed lactic acid (3.89 g/L) and acetic acid (0.49 g/L) as the main SMPs. The enzymatic analysis for α-amylase and amyloglucosidase showed maximum activities 4.8 U/ml and 4.53 U/ml at 96 h, respectively (Fig. 4A). Interestingly, throughout the fermentation with food waste no significant difference between the activities of amylase and amyloglucosidase was observed that was

Table 2
Carbon mole balance for the fermentation by *G. thermoglucosidasius* using glucose, xylose and starch.

Carbon Balance*									
Substrate/metabolite	Glucose			Xylose			Starch		
	mM	C-mM	%	mM	C-mM	%	mM	C-mM	%
Substrate utilized	107.8 ± 3.01	646.8 ± 18.11	100 ± 2.79	133.3 ± 3.46	666.5 ± 17.4	100 ± 2.6	199.8 ± 6	1198.8 ± 36	100 ± 4.42
Biomass	162 ± 4.7[#]	162 ± 4.7	25 ± 0.76	134.2 ± 3.76	134.2 ± 3.76	20.1 ± .81	99.5 ± 2.79	99.5 ± 2.79	8.3 ± .26
Glucose	–	–	–	–	–	–	52 ± 1.37	624 ± 17	52.1 ± 1.6
Ethanol	40 ± 1.01	80 ± 2.2	12.7 ± .36	26 ± 0.98	52 ± 1.5	7.8 ± .32	78.14 ± 2.34	156.3 ± 3.9	13 ± .36
Acetate	37.5 ± 1.16	75 ± 2.1	11.6 ± .34	20 ± 0.56	50 ± 1.34	7.5 ± .29	8.29 ± .24	16.8 ± 0.49	1.4 ± 0.05
Lactate	75 ± 2.25	225 ± 6.75	34.7 ± 1.09	60 ± 1.68	180 ± 5.04	27 ± 1.09	20.5 ± 0.636	61.6 ± 1.85	5.13 ± 0.17
Formate	–	–	–	–	–	–	21.4 ± 0.6	21.4 ± 0.6	1.79 ± 0.05
Propionate	–	–	–	–	–	–	0.05 ± 0.0013	0.14 ± 0.0042	0.01 ± .0004
Succinate	–	–	–	–	–	–	0.48 ± 0.0134	1.9 ± 0.053	0.16 ± 0.005
CO_2	75 ± 2.48	75 ± 2.48	11.6 ± .4	49.3 ± 1.52	49.3 ± 1.52	7.4 ± 0.33	110 ± 3.85	110 ± 3.85	9.2 ± .35
Total	–	619 ± 18.6	–	–	465.5±	–	–	1091.6 ± 32.3	–
Recovery	–	–	95.7 ± 2.95	–	–	69.8 ± 2.84	–	–	91 ± 2.64

*Calculated by converting the moles into carbon moles. A carbon mole is that amount of substance which contains 1 mol of elemental carbon.
#calculated using formula $CH_{1.8}O_{0.5}N_{0.2}$ [20].

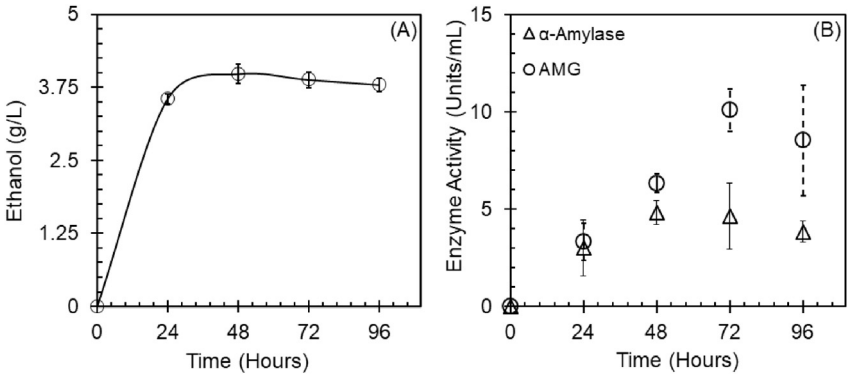

Fig. 2. Ethanol production (A) and enzyme activities (B) using starch as a substrate by *G. thermoglucosidasius*. The values are mean of triplicates with error bars showing the standard deviation.

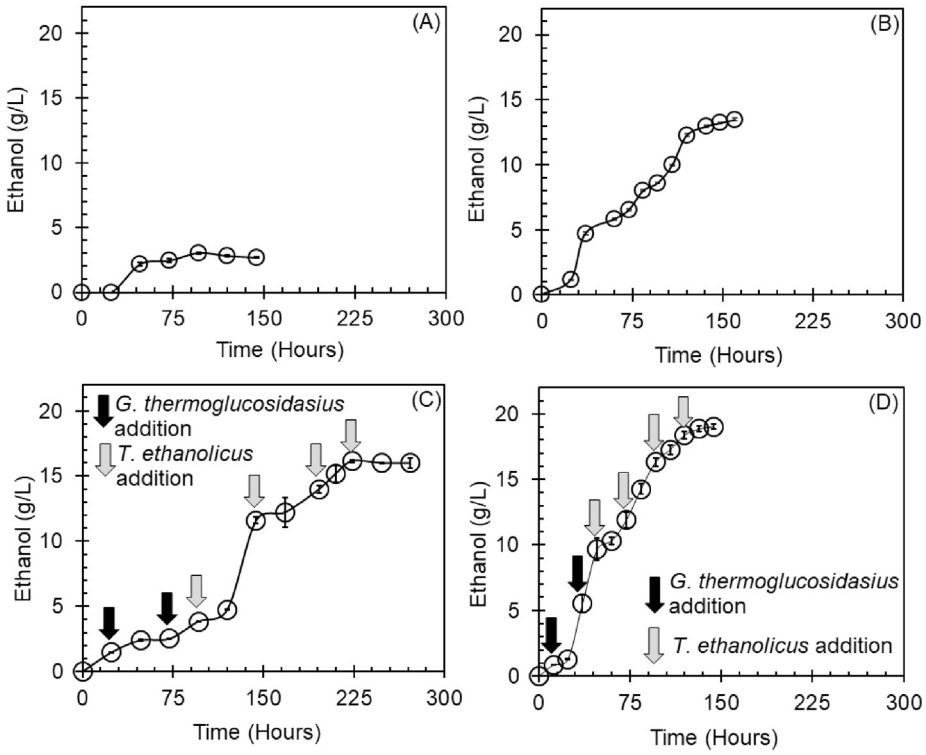

Fig. 3. Ethanol production with (A) 10% (w/v) food waste in 500 mL serum bottles, (B) 10% (w/v) food waste in 1L bioreactor, (C) 20% (w/v) food waste in 1L bioreactor during sequential cultivation and (D) 20% (w/v) food waste in 40L bioreactor during sequential cultivation. The values are mean of triplicate runs with SD ± 3.

seen during fermentation with starch.

When pure starch was used as a substrate the fermentation stopped after 48 h. On the contrary with food waste, the fermentation continued after 48 h and ethanol, lactate, and acetate reached their maximum values at 96 h. The amount of ethanol, lactate, and acetate decreased (ethanol) or remained constant (lactate and acetate) after 96 h. At the end of the fermentation a very small amount of glucose was observed. This suggested that glucose produced by food waste hydrolysis was simultaneously utilized for growth and fermentation. However, no plausible reason could be made for why the ethanol and SMPs production stopped after 96 h.

In 1L DASGIP reactor: In 1L DASGIP reactor, with 10% (w/v) of food waste, an ethanol yield of 13.5 g/L was obtained in 168 h (Fig. 3B). The main SMPs were acetate (1.46 g/L), lactate (4.29 g/L) and propionate (1.02 g/L). The ethanol production in the 1L bioreactor started before 24 h compared to the serum bottles where no ethanol was observed within 24 h. With bioreactor an early ethanol production was observed due to more control on the fermentative conditions such as pH, agitation, and gas sparging etc. It has been shown previously that better mixing improves the mass transfer, cell growth and ethanol productivity [27]. After 132 h the ethanol production remained constant with very negligible changes in the production. Also, no further increase in the concentration of the SMPs was also observed. This could be the result of *G. thermoglucosidasius* entering late stationary or death phase, where all the cellular activity stops. The negligible amounts of increase could be because of the release of the intracellular ethanol and other SMPs after cell death.

The activities for enzymes α-amylase (18.5 U/ml) and amyloglucosidase (19.4 U/ml) also increased during the fermentation in DASGIP reactor (Fig. 4B). Interestingly, the enzyme activities

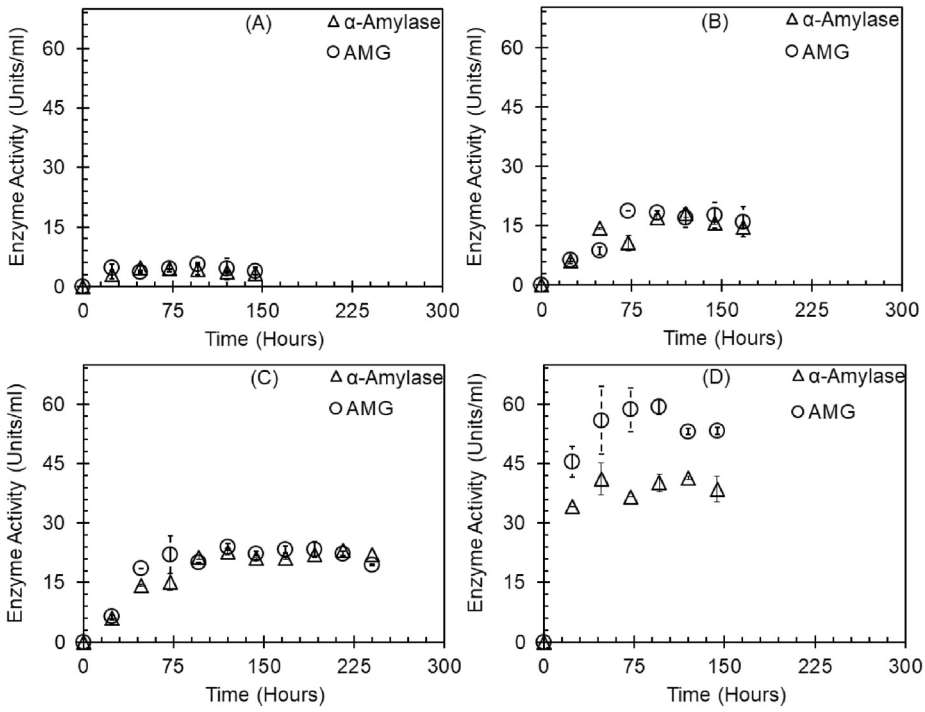

Fig. 4. Enzyme activity (amylase and amyloglucosidase) during fermentation with (A) 10% (w/v) food waste in 500 mL serum bottles, (B) 10% (w/v) food waste in 1L bioreactor, (C) 20% (w/v) food waste in 1L bioreactor during sequential cultivation and (D) 20% (w/v) food waste in 40L bioreactor during sequential cultivation. The values are mean of triplicate runs with SD ± 3.

showed a variable pattern during the fermentation with a maximum difference observed at 72 h among the two enzymes. At 48 h higher amylase activity results in the formation of more maltooligosaccharides that are inhibitory to the amylase enzymes [25]. Hence, at 72 h amylase activity decreased and amyloglucosidase activity increased. However, after 72 h the enzyme activities again levelled until the end of the fermentation i.e. 168 h.

3.3.2. Sequential cultivation of the thermophilic microorganisms

With 10% (w/v) food waste, *G. thermoglucosidasius* produced 13.5 g/L in the 1L DASGIP bioreactor. However, further increase in the food waste to 20% (w/v) did not increase the ethanol production. To increase the ethanol production with increased substrate concentration (20% w/v), sequential cultivation of two thermophiles *G. thermoglucosidasius* and *T. ethanolicus* was carried. *T. ethanolicus* is a thermophilic non-spore forming obligate anaerobic bacteria capable of growing at a temperature of 37–78 °C and is able to ferment >20% (w/v) of sugars without inhibition [28]. During sequential cultivation *T. ethanolicus* was added 96 h after the inoculation of *G. thermoglucosidasius*. After the addition of *T. ethanolicus* increase in ethanol production was observed with a cumulative yield of 7.46 g/L in 160 h. The addition of *T. ethanolicus* increased the ethanol production from 6.51 to 7.46 g/L in 224 h. However, further incubation did not result in any increase in the ethanol yield, but on the contrary reduced it to 7.21 g/L at 272 h.

It had been shown previously that *T. ethanolicus* cannot produce ethanol under certain conditions such as high sugar content (>20% w/v), pH > 4.2 or high ethanol concentration (>2.5% v/v) [28,29]. However, none of these conditions were encountered during the fermentation ruling out the possibility of these affecting the fermentation and ethanol production [28]. Wiegel and Ljungdahl [28] had described that longer incubation of *T. ethanolicus* cells at higher temperature causes the cells to loose viability and suggested to sub-culture the cells after 72 h to maintain the cell viability. In our case also this appeared as a plausible reason for reduced ethanol production over longer incubation period. Hence, *T. ethanolicus* cells were added after regular intervals of 48 h (after 96 h) to maintain the cell viability and fermentation capability. The addition of *T. ethanolicus* cells increased the ethanol production with a cumulative yield of 10.31 g/L in 240 h. The ethanol production improved from 7.21 g/L to 10.31 g/L with the addition of *T. ethanolicus* cells. However, the ethanol production was still less than 13.5 g/L obtained with *G. thermoglucosidasius*.

The HPLC analysis showed that after 210 h glucose started accumulating in the fermentation broth. No glucose was observed during the fermentation until t = 210 h suggesting that the cells did not utilize and ferment the available sugar. It was hypothesized that the cells were limited by the absence of certain nutrients as all other fermentation limiting conditions (pH, sugar and ethanol concentration, and cell viability) mentioned previously were not encountered. Hence, 20 ml (5X) of the nutrient media (devoid of any carbon and nitrogen source) was added to the reaction system along with *T. ethanolicus* cells after 48 h. The addition of the media increased the ethanol production from 11.5 g/L to 12.4 g/L.

To further improve the ethanol production cell addition for both *G. thermoglucosidasius* and *T. ethanolicus* was carried. The cells of *G. thermoglucosidasius* were added every 24 h from t = 0 h and cells of *T. ethanolicus* were added at every 48 h from t = 96 h. Along with the cells of *T. ethanolicus* 20 ml (5X) of media was also added (Fig. 3C). The addition of the *G. thermoglucosidasius* cells after 24 h did not result in the increase of ethanol yield rather decreased it from 5.32 g/L to 3.84 g/L. On the other hand, addition of *T. ethanolicus* cells after 96 h increased the ethanol production from 3.84 g/L to16.1 g/L in 128 h. Interestingly, with the addition of *G. thermoglucosidasius* cells the ethanol production increased sharply after the first addition of *T. ethanolicus* cells. This can be due to the increased availability of hydrolysable sugars carried by increased cells of *G. thermoglucosidasius*. The ethanol production

increased to a final concentration of 16.1 g/L in 224 h with an overall ethanol productivity of 0.072 g/L/h. The ethanol productivity was however lower than that observed with *G. thermoglucosidasius* (0.1 g/l/h). Further, addition of the *T. ethanolicus* cells did not show any increase in the ethanol production. Other literature studies have also shown that co-culture of microbes can increases the ethanol production [9]. [39] also reported an ethanol yield of 1.25 g/L with 1% (w/v) of bean curd refuse with sequential cultivation of a *Geobacillus* and *Thermoanaerobacter* spp. The co-cultivation of *Thermoanaerobacterium saccharolyticum* and *Clostridium thermocellum* growing at 60 °C increased gave an ethanol yield of 38.1 g/L with avicel [30]. A sequential processing with *Caldicellulosiruptor kronotskyensis* and *Cuproadidus necator* increased the production of polyhydroxy butyrate by 9 folds compared to the monoculture of *Caldicellulosiruptor kronotskyensis* [31]. The two organisms respectively grew at 75 °C and 30 °C temperatures. A temperature difference at different process stages can increase the capital and operational costs at an industrial scale. The current study is the first report of ethanol production by sequential cultivation of *G. thermoglucosidasius* and *T. ethanolicus* using food waste and scale up to 1L reactor.

HPLC analysis showed that both the organisms produced lactic acid, acetic acid and propionic acid as the main soluble metabolic products (SMP). The addition of *G. thermoglucosidasius* cells at 24 h interval resulted in more lactic acid (6.5 g/L), and acetic acid (3.9 g/L). As the fermentation with *G. thermoglucosidasius* ended, the lactic acid amount got reduced. As the *T. ethanolicus* cells were added the amount of SMPs increased with lactate (4.32 g/L), and acetate (3.6 g/L) as the main SMPs (Table 3). An increase in the amount of glucose, and xylose was observed at 48 h which could be due to the improved hydrolysis and more sugar release after first addition of *G. thermoglucosidasius* cells.

Mass balance for the ethanol production in the serum bottles and 1L bioreactor with food waste is shown in Table 3. The food waste utilized was recovered as sugars, ethanol and other SMPs. It was observed that the use of bioreactor significantly improved the mass recovery during the fermentation. In serum bottles only 39.2% of the substrate utilized could be recovered, whereas with the use of bioreactor the recovery increased to 92.8% with *G. thermoglucosidasius.* The mass recovery, however, decreased to 87.5% when sequential cultivation of *G. thermoglucosidasius* and *T. ethanolicus* was done. However, it is worthwhile mentioning here that during sequential cultivation 20% (w/v) of food waste was used. It is recommended that for a successful industrial operation a higher percentage of substrates are used [32]. Thus, taken into consideration that higher substrate concentration can be hydrolyzed and fermented the sequential cultivation holds the potential to be used at an industrial scale.

At the end of the fermentation no glucose was observed but, other sugars galactose, fructose and arabinose were also observed (Table 3). These sugars were absent in the t=0 samples when no inoculum was added. It is worth to mention here that these sugars can be produced by the biochemical transformation of glucose catalyzed by both enzymes and chemical catalysts. It had been reported previously that glucose isomerase enzymes are capable of converting glucose to fructose and the rate increase with the presence of certain metal ions viz. Mg^{+2}, and B^{+3} etc., and higher temperature example 60 °C [33,34]. These conversion can also occur via Lobry de Bryun-Alberda Van Ekenstein Transformation (LBET) where in the presence of base example NaOH, the glucose can epimerize to its isomer galactose, fructose and mannose [35]. Hence, the transformation of the glucose into these isoforms can be considered as the result of simultaneous chemical and biochemical transformation in the presence of the appropriate catalysts.

3.4. Scaling up of ethanol production to 40L bioreactor-

The fermentation in the 40L bioreactor was carried in a total volume of 20L (18.72 L media+1 L inoculum+100 ml urea+100 ml trace metal solution +10 ml biotin+10 ml) at 60 °C and variable agitation speeds. With food waste an ethanol yield of 18.4 g/L of ethanol was obtained in 120 h (Fig. 3D). The fermentation was carried by sentential cultivation of *G. thermoglucosidasius* and *T. ethanolicus.* 9.7 g/L of ethanol was produced in 48 h with *G. thermoglucosidasius.* Further inoculation of *T. ethanolicus* after 48 h increased the ethanol production to 18.4 g/L in 120 h. The cell addition (4 g/L) of *G. thermoglucosidasius* was carried at t = 12 and 36 h. Similarly, the cell addition of *T. ethanolicus* (6 g/L) was carried at t = 48, 72, 96 and 120 h 50 ml (20X) media was also added with the addition of *T. ethanolicus* cells while no media was added with the addition of *G. thermoglucosidasius* cells.

A maximum ethanol production of 18.4 g/L was obtained with a productivity of 0.15 g/L/h. The ethanol produced was comparable to several other pilot scale studies that carried ethanol production with non conventional substrates. Skiba and coworkers reported a yield of 16.6 g/L with oat hulls [36] and Saha and coworkers (2015) reported an ethanol concentration of 34.4 g/L with pretreated wheat straw [37]. However the ethanol productivity was lowest when compared to the several other studies. The ethanol productives ranged from 0.22 g/L/h [38] to 9.7 g/L/h [39]. Further, the ethanol yield 0.24 g/g sugar consumed was comparable to the other studies carried by Ref. [40]; where a yield of 0.26 g/g consumed sugar was obtained and [36]; who reported a yield of 0.25 g/g consumed sugars. However, other studies have also reported a near theoretical yield approaching 0.51 g/g sugar consumed [37]. The lower productivity was observed as the organisms used in the present study were wild type and no pretreatment was done, whereas the other studies involved pretreatment or recombinant bacteria/yeast or both.

A maximum activity of 41.4U/ml and 59.2U/ml respectively was obtained for amylase and amyloglucosidase during fermentation in 40L bioreactor (Fig. 4D). No significant change was observed in the enzyme activities during the fermentation. At 72 h though, a reduction in the amylase activity was observed. The amyloglucosidase activity was greater than amylase throughout the fermentation. Similarly, a decrease in the amyloglucosidase activity was observed at 120 h. A higher amyloglucosidase is also preferred in a enzyme based pretreatement method [27]. It had been shown previously also that presence of the amylase enzymatic activity in the wild type strain omitted the required of pretreatment. *Wickeremia* sp. produced 21.7 g/L of ethanol from potato peel waste [41]. No pretreatment was give to the substrate due to the ability of the wild type strain to produce amylase (4900 U/ml) and glucoamylase enzymes (2.82 U/ml). Another reported isolate *Scheffersomyces shehatae* was also able to produce ethanol (9.21 g/L) from stacrh without any pretreatement as it produced 21 U/ml and 32 U/ml respectively of amylase and glucoamylase [26]. Hence, the native ability of the ethanologens to produce the required hyrolytic enzymes can improve the currently used bioprocesses for ethanol production.

Post fermentation analysis of the solid and liquid residue showed that no sugar was observed in the fermentation broth. This signified that all the available sugar was utilized for cell growth, cell maintainence and fermentation. However, a small change was observed in the cellulosic and hemicellulosic content of the food waste post fermentation. The analysis of the solid residue showed presence of 15.7 ± 0.62% (w/v) cellulose and 2.92 ± 0.06% (w/v) hemicellulose. The enzymatic analysis for cellulase and hemicellulase did not show any enzymatic activity. The change observed in the cellulosic and hemicellulosic content can be due to the

Table 3
Mass balance of ethanol production by *G. thermoglucosidasius* with 10% (w/v) food waste in serum bottles and 1L DASGIP bioreactor and with 20% (w/v) food waste in 1L DASGIP bioreactor and 40L BioFlo 510 bioreactor with *T.* ethanolicus, at 60 °C. The values are mean of triplicate runs with SD ± 3.

Mass balance												
	G. thermoglucosidasius			*G. thermoglucosidasius*			*G. thermoglucosidasius* and *T. ethanolicus*			*G. thermoglucosidasius* and *T. ethanolicus*		
	Serum bottles (500 mL)			Bioreactor (1L)						Bioreactor (40 L)		
	10% w/v (food waste)			10% w/v (food waste)			20% w/v (food waste)			20% w/v (food waste)		
Substrate/ metabolite	mM	Mass*	%	mM	Mass	%	mM	Mass	%	mM	Mass	%
Substrate utilized	191.7 ± 5.01	31.05 ± .27[#]	100 ± 2.61	191.7 ± 6.24	35.05 ± 0.88	100± 3.26	350 ± 10.9	62.9 ± 1.53[$]	100 ± 3.11	383.4 ± 12.3	64.78 ± 2.11[$]	100± 4.1
Biomass										–	–	–
Glucose	–	–	–	5.9± 0.21	1.06 ± 0.03	3± 0.082	17.7 ± 0.55	3.2± 0.067	5.2± 0.12	–	–	–
Maltose	–	–	–	–	–	–	–	–	–	3.5 ± 0.09	1.2± 0.027	1.73± 0.045
Mannose	–	–	–	0.08 ± 0.0002	0.01 ± 0.0002	0.03± 0.0006	7.22 ± 0.16	1.3 ± 0.034	0.24± 0.061	–	–	–
Galactose	2.5± .016	0.45 ± .016	1.5 ± 0.13	0.61 ± 0.003	0.11 ± 0.002	0.31± 0.0061	11.1 ± 0.2	2± 0.053	0.35± 0.1	23.3 ± 0.57	4.2± 0.13	6.04± 0.21
Xylose	–	–	–	–	–	–	11.2 ± 0.24	1.7± 0.05	2.73± 0.09	–	–	–
Fructose	–	–	–	0.28 ± 0.014	0.05 ± 0.001	0.14± 0.0031	0.9 ± 0.023	0.89± 0.027	0.16± 0.05	6.7 ± 0.201	1.2± 0.028	1.73± 0.047
Arabinose	–	–	–	0.12 ± 0.004	0.02 ± 0.0009	0.06± 0.0028	1.6 ± 0.025	0.24± 0.0077	0.4± 0.014	2.6 ± 0.05	0.4± 0.011	0.58± 0.019
Ethanol	65.8± 2.1	3.03 ± .084	9.8 ± .70	282 ± 18.2	13 ± 0.41	37± 1.25	350 ± 10.1	16.1± 0.52	29.3± 0.94	400 ± 14	18.4 ± 0.57	30.9± 1.01
Acetate	8.3± 0.24	0.49 ± .055	1.6 ± 0.45	24.7 ± 0.82	1.46 ± 0.37	4.2± 1.13	61± 1.8	3.6± 0.016	5.8± 0.20	50.8 ± 1.32	3± 0.05	4.32± 0.089
Lactate	43.2± 1.1	3.89 ± .074	12.5 ± 0.37	50± 1.6	4.3 ± 0.14	12.3± 0.43	48± 1.15	4.32± 0.018	7.84± 0.25	46.1 ± 1.11	4.15 ± 0.083	6± 0.15
Propionate	–	–	–	1.5 ± 0.038	0.11 ± 0.0033	0.31± 0.01	3.8 ± .12	0.28± 0.0062	0.52± 0.011	14 ± 0.4	1.04 ± 0.026	1.5± 0.046
Formate	–	–	–	–	–	–	56.4 ± 1.7	2.6± 0.016	4.72± 0.01	48.9 ± 2,2	1.2 ± 0.0264	1.85± 0.047
Succinate	–	–	–	1.2 ± 0.028	0.14 ± 0.0028	0.4± 0.009	8.6 ± .27	1.01± 0.026	1.6 ± .024	35.6 ± 1.75	4.2 ± 0.12	6.04± 0.021
CO_2	98± 3.67	4.3± 0.054	13.8 ± 0.44	326 ± 10.1	12.3 ± 0.36	35.1± 1.11	404 ± 11.7	17.8± 0.59	28.7± 1.07	466 ± 21.2	20.5 ± 0.62	29.5± 1.10
Total		12.16 ± 0.36			32.56 ± 0.98			55.04 ± 1.5		–	59.4 ± 1.9	–
Recovery			39.2 ± 1.71			92.8± 2.83			87.5± 2.94	–		91.9± 2.64

*g/L.
#Glucose equivalent calculated based on the starch sugars in the blended food waste on dry basis.
$Glucose and xylose equivalent calculated on the base of the utilization of starch sugars and reduction in the cellulosic and hemicellulosic content on dry basis.

addition of acid and base in the fermentation media to adjust the pH change. It had been shown previously that acid and alkali treatment respectively target the hydrolysis of cellulosic and hemicellulosic content [11]. As per calculations about 2.2L of 10 N NaOH and 0.25L of 0.1N HCl was consumed during the fermentation for pH adjustment. One can righteosuly argue here that such volumes and strengths respectively of the acid and base should hydrolyze the cellulosic and hemicellulosic content completely. However, two reasons (1) shorter residence time with acid and base and (2) different composition of the substrate for pretreatment, nullify the argument. It had been shown that acidic pretreatments are carried at higher temperatures (130–200 °C) with concentrations ranging from 0.05 to 8% (w/v) of acid [11,46]. A dilute acid pretreatment of food waste was carried with 0.8% (w/v) of H_2SO_4 at 160 °C for 130 min [42]. Similarly, alkali pretreatment also require higher temperature and longer residence time. Sugarcane baggase was pretreated with 1.8% (w/v) of NaOH solution at 110 °C for 60 min to obtaine sugars for ethanol production [43]. Also, the substrate for pretreatment is free from organic acids such as lactic acid, acetic acid etc. that react with acid and base to adjust the pH during the fermentation. Hence, the small change observed in the cellulosic and hemicellulosic content can be corelated to hydrolysis with acid and base addition. The absence of hydrolysis of cellulosic and hemicellulosic also resulted in reduced availability of sugars and hence lower ethanol production.

The HPLC analysis of the fermentation broth showed the presence of carbohydrates viz. maltose, galactose, mannose, fructose, glucose and arabinose etc., and organic acid viz. succinic acid, acetic acid, lactic acid, fomric acid, and propionic acid etc. The maltose was observed in the broth after 12 h and reached to a maximum of 12 g/L in 24 h (Fig. 5). After this the amount of maltose decreased till 72 h. At 72 h 2.16 g/L of maltose was observed and no significant maltose was observed till the end of the fermentation. No glucose was observed throughout the fermentation except at 48 h where 2.85 g/L of glucose was observed. Fig. 5 shows the production profile of lactate, acetate, maltose and glucose during the fermentation. The main organic acids observed during the fermentation were lactic acid, acetic acid, propionic acid, succinic acid.

The mass balance analysis of the fermentation with food waste showed that the 91.9% of the substrate utilized by the microbes was recovered during fermentation in 40L bioreactor as metabolites and leftover sugars (Table 3). The recovery of 91.9% of mass depicted

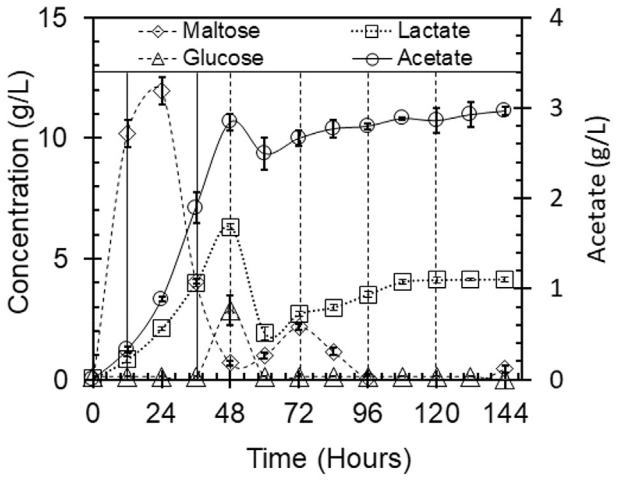

Fig. 5. The production profile of acids and sugars during the sequential fermentation of food waste by *Geobacillus thermoglucosidasius* and *Thermoanaerobacter ethanolicus* at 60 °C and pH 6.75 in 40L bioreactor. The solid straight lines represent the point of addition of *Geobacillus thermoglucosidasius* and dotted straight lines represent the point of addition of *Thermoanaerobacter ethanolicus*. The values are the mean of triplicate runs with standard deviation ± 3.

that the process was efficient in terms of recovering the input mass. The recovery percentage was in coherence with other studies that also showed high recovery of the input substrate in terms of sugars and metabolites. Dhiman and coworkers (2017) also showed 94.2% recovery in terms of C moles from food waste during ethanol production with the food waste [15].

Energy balance showed that only 1.67% of the energy in food waste was transformed into bioethanol. However, when compared to the literature studies the energy efficiency appeared reasonable. Yan and coworkers obtained 96.4 g/L of ethanol with 200 g/L of sugar that corresponds to an energy efficiency of 0.90% [44]. Similarly, an energy conversion of 1.54% was observed with enzymatically hydrolyzed food waste that gave 58 g/l of ethanol from 127 g/L of glucose [27]. Based on the scale up studies, it was determined that using the developed sequential cultivation process 105.8L of ethanol can be obtained per ton (US ton) of the food waste. This was equivalent to the 70.1L of gasoline equivalent. The scale up of the process from 1L to 40L increased the gasoline equivalent from 64.1L to 70.1L. The reported gasoline equivalent was lower than reported by Mahmoodi and coworkers (2018) who reported a gasoline equivalent of 326.6L after fermentation of per metric ton of organic fraction of municipal food solid waste [45]. The higher gasoline equivalent was observed because the substrate was acid treated and the waste residue from ethanol production was used for methane production. This study was based on the fermentation and utilization of only starchy part of the food waste. If the cellulosic and hemicellulosic portions can also be utilized the process can be improved further. Further, the integration of the current process with methane production using the left-over waste can also increase the gasoline equivalent and make it a success at industrial scale.

4. Conclusions

G. thermoglucosidasius produced 13 g/L of ethanol from 10% (w/v) of untreated food waste (dry basis). The sequential cultivation of the thermophiles improved the ethanol production giving a final yield of 16.1 g/L in 1L bioreactor with 20% (w/v) of food waste. An overall 87.5% of the input mass was recovered at the end of fermentation. Scaling up to 40L gave 18.4g/L ethanol with 92% recovery of the substrate mass fed into the reactor. The complete utilization of the sugars depicted the suitability of the process. The obliteration of the pretreatment substantiates environment friendly nature of the process. Further, process development to include microorganisms that can hydrolyze cellulose and hemicellulose portions and integration of the process to other bioprocesses such as biogas production can aid in higher energy recovery from the food waste with overall process improvement.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Mohit Bibra: Data curation, Validation, Writing - original draft. **Navanietha K. Rathinam:** Data curation, Validation. **Glenn R. Johnson:** Writing - review & editing, Supervision. **Rajesh K. Sani:** Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2020.02.093.

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