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Research Article

Isolation and Identification of Cellulase Producing and Sugar Fermenting Bacteria for Second-Generation Bioethanol Production

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ABSTRACT. Over the last decades, the negative impacts of fossil fuel on the environment and increasing demand for energy due to the unavoidable depletion of fossil fuels, has transformed the world's interests towards alternative fuels. In particular, bioethanol production from cellulosic biomass for the transportation sector has been incrementing since the last decade. The bacterial pathway for bioethanol production is a relatively novel concept and the present study focused on the isolation of potential "cellulase-producing" bacteria from cow dung, compost soil, and termite gut and isolating sugar fermenting bacteria from palm wine. To select potential candidates for cellulase enzyme production, primary and secondary assays were conducted using the Gram's iodine stain in Carboxy Methyl Cellulose (CMC) medium and the Dinitrosalicylic acid (DNS) assays, respectively. Durham tube assay and Solid-Phase Micro-Extraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC-MS) was used to evaluate the sugar fermenting efficiency of the isolated bacteria. Out of 48 bacterial isolates, 27 showed cellulase activity where *Nocardiopsis* sp. (S-6) demonstrated the highest extracellular crude enzyme activity of endoglucanase (1.56 ±0.021 U) and total cellulase activity (0.93 ±0.012 U). The second-highest extracellular crude enzyme activity of endoglucanase (0.21 ±0.021 U) and total cellulase activity (0.35 ±0.021 U) was recorded by *Bacillus* sp. (T-4). Out of a total of 8 bacterial isolates, *Achromobacter* sp. (PW-7) was positive for sugar fermentation resulting in 3.07% of ethanol in broth medium at 48 h incubation. The results of the study revealed that *Nocardiopsis* sp. (S-6) had the highest cellulase enzyme activity. However, the highest ethanol percentage was achieved with by having both *Bacillus* sp. (T-4) and *Achromobacter* sp. (PW-7) for the simultaneous saccharification and fermentation (SSF) method, as compared to separate hydrolysis and fermentation (SHF) methodologies.

Keywords: Bioethanol; Carboxy Methyl Cellulose; Cellulase producing bacteria; Solid Phase Micro-Extraction; Sugar fermenting bacteria

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1. Introduction

Increasing energy demand stemming from a growing population is one of the major challenges facing mankind at present (Szilvia *et al.* 2015). Total world energy supply via fossil fuels (oil, coal, and gas) represents 80% of energy sources, followed by renewables and nuclear energy; 18% and 2% respectively (WBA Global Bioenergy Statistics, 2018). Since fossil fuel is non-renewable and finite in quantity, scientists found that fossil fuel reserves of oil, natural gas, and coal will be depleted in approximately 35, 37, and 107 years respectively (Madusanka & Manage, 2018). Therefore, the world is moving towards renewable energies such as biofuel (Madusanka & Manage, 2018), hydropower (Balkhair & Rahman, 2017), wind (Zeng *et al.* 2019), tidal and waves (Niegel, 2018), and Ocean Thermal Energy Conversion (OTEC) (Ahmadi, Dincer & Rosen, 2015), etc. due to having lower environmental impacts, reliability and sustainable supply (Madusanka & Manage, 2018).

Sri Lanka is completely dependent on importation of petroleum products to meet fuel requirements. Due to the current depletion of fossil fuels, there is a growing interest to discover renewable, abundant and economically-favourable alternatives. Considering the infrastructure cost, energy generation rate, low hydropower generation during adverse monsoon seasons and consistent decline of foreign direct investment (FDI) in the power sector, biofuels, especially bioethanol is considered as one of the successful alternative energies for Sri Lanka (Sri Lanka Development Update, 2017).

However, there have been a handful of studies (Kularathne *et al.* 2020; Jayasekara, Abayasekara & Ratnayake, 2019; Jayathilaka *et al.* 2018; Thakshika, Peries & Henegamage, 2019; Senarathna, Rupasinghe &

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Bandara, 2019) carried out in Sri Lanka regarding bioethanol production through the biological pathway and cost-effective processes for converting cellulosic biomass to fuels are yet to be fully realized. Approximately 12 million litres of ethanol are being produced using sugar-cane molasses in Sri Lanka. However, limited molasses is available to supply energy requirements (Gunawardena, 2009). Due to relatively high population density, limited land and cultivation of food crops instead of fuel can be seen as potential hurdles. Thus, the present study was carried out to initiate the production of second-generation bioethanol using cellulosic biomasses.

Cellulose is the most abundant biopolymer in nature, which consists of the major structural polymer (40-50%) in plant cell walls. Cellulose has the potential to produce bioethanol as an alternative energy fuel for the current fuel crisis in the world (Aditiya *et al.* 2016; Song *et al.* 2018). Bioethanol is the highest produced liquid biofuel in the world via the biomass fermentation process (Rastogi & Shrivastava, 2017). Currently, the majority of the ethanol plants across the world are first-generation, including, corn kernels (starch) in the US and sugarcane (sucrose) in Brazil (Mohanty & Swain, 2019; Liu *et al.* 2019). The use of edible crops as energy sources causes the "food vs fuel" debate. Thus, the second-generation biofuels derived from non-food cellulosic biomass have growing interest due to having sustainable, cheap and abundant feedstock and due to bioethanol being produced at reasonable costs (Hassan, Williams & Jaiswal, 2019).

Bioethanol production is a natural process of sugar fermentation into ethanol by microorganisms. However, the production of bioethanol as a fuel has recently gained increasing interest worldwide. Fuel ethanol differs from that of alcoholic beverages due to the need for rapid, complete and more profitable conversion (Westman and Franzén, 2015).

The steps of bio-ethanol production through biological pathway mainly include the conversion of cellulosic biomass into fermentable sugar called 'hydrolysis or saccharification' which facilitate the dissolution of the cellulose into simple sugar monomers (glucose) and the conversion of sugar into ethanol called 'sugar fermentation' (Weerasinghe, Madusanka, and Pathmalal, 2019). Hydrolysis can be catalyzed by a highly specific cellulase enzyme complex which consists of endoglucanase, exoglucanase, and ß-glucosidase which hydrolyzed complex cellulose polymer into simple glucose monomers (Lu *et al.* 2019).

Microorganisms play a significant role in both cellulase enzyme production and the sugar fermentation process (Zhao *et al.* 2016). Therefore, the selection of suitable microbial strains that have the potential to produce a high amount of cellulase and consumption of pentoses and hexose sugars for ethanol production is important (Azhar *et al.* 2017). High tolerance against ethanol, inhibitory compounds and the requirement of minimum nutrients are the factors to be considered to selecting suitable microorganisms (Magocha *et al*. 2018).

A diverse group of microorganisms; fungi (Nair & Taherzadeh, 2016), bacterial (Zhao *et al*. 2016), and yeast (Azhar *et al.* 2017) has been reported for cellulosic activities and fermentation processes. Cellulosic enzyme activity by fungus; *Fusarium* sp.*, Trichoderma* sp.*, Penicillium* sp.*, Aspergillus* sp., etc. (Sarsaiya *et al.* 2018; Tsegaye, Balomajumder & Roy, 2019), bacteria; *Pseudomonas* sp.*, Streptomyces* sp.*, Clostridium* sp.*, Actinomycetes* sp.*, Bacillus* sp., etc. (Rajwade, Paknikar & Kumbhar, 2015; Anu *et al.* 2020) has been recorded as potential candidates. The *Saccharomyces cerevisiae* is the most-commonly used microorganism where several other fungal species belong to genera *Fusarium*, *Rhizopus, Mucor* and bacteria; *Escherichia coli*, *Klebsiella oxytoca,* and *Zymomonas mobilis* have been recorded for the sugar fermentation step in bioethanol production (Sarkar *et al*. 2020; Liu *et al*. 2019). It has been recorded that bacteria have a high growth rate, high adaptability in any environmental conditions, and the ability to produce highly stable enzyme complement, thus possessing good potential to be used in bioethanol production than fungal counterparts (Yadav, 2017).

Thus, the objectives of this study were, to isolate and to screen bacteria for the production of cellulase and sugar fermentation with optimizing different nutritional and environmental parameters for maximum cellulase production and sugar fermentation as an eco-friendly alternative green approach as a potential solution to the future energy crisis.

2. Materials and Methods

2.1 Isolation of cellulase producing bacteria

Compost soil and freshly dumped cow dung samples were collected using a sterilized spatula into sterile containers and stored at 4˚C for isolation of bacteria. 1 g of compost soil and cow dung were serially diluted in 0.9% sterile saline solution up to six-fold dilution (10^{-6}). Thereafter, 1 mL of each dilution was introduced to sterilized petri plates followed by standard pour plate technique (Idroos & Manage, 2018; Liyanage & Manage, 2016a) using the Carboxyl Methyl Cellulose(CMC) agar media which contained; cellulose substrate(CMC), 2 gL^{-1}; $MgSO_4.7H_2O$, 0.25 gL^{-1}; K_2HPO_4, 0.5 gL^{-1}; peptone, 2 gL^{-1}; bacteriological agar, 15 gL^{-1}. All analytical grade chemicals were purchased from Sigma Aldrich, USA. After 48 h of incubation at 28 ±2˚C, bacterial colonies with different colony and morphological characteristics were selected for isolation and repeated streaking on CMC agar plates was performed to obtain pure cultures (Liyanage & Manage, 2016b) which was followed by sub-culturing and storage in agar slants at -20˚C in lysogeny agar-glycerol media for future studies (Ekanayake & Manage, 2020).

Isolation of bacteria from termite gut was performed by sterilizing 30 living termites by submergence in 70% ethanol for 3 min followed by rinsing in sterile water twice, prior to dissection. Termites were de-headed and abdominal parts were transferred to 1.5 mL micro-centrifuge tubes containing 0.5 mL of sterile distilled water; samples were squeezed using a pestle and then 1 mL of Phosphate Buffer Solution (PBS) was added and subjected to vortex for 5 min. To separate the microbiota from the gut content, the sample was centrifuged (Model: MSE MINOR 35 YM 694, England) at 5000 rpm at 4˚C for 15 min. Then 1 mL of supernatant was subjected to a dilution series and followed by bacteria isolation (Phuog, Lena & Thang, 2015). Cellulase producing bacteria isolated from cow dung, compost soil and termite gut were

referred to as CD (e.g. CD-14), S (e.g. S-6) and T (e.g. T-1) respectively.

2.1.1 Primary screening of cellulase producing bacteria

Isolated pure bacterial cultures were equalized using 0.5 McFarland standards in 0.9% saline water to obtain a similar number of (1.5´ 10^8) colony forming units (CFU mL^{-1}). Following which, CMC broth was added and incubated for 48 h at 25 ±1˚C in the shaker (Model: Multishaker MMS, Japan) at 100 rpm. Subsequently, cultures were centrifuged at 5000 rpm at 4˚C for 15 min to obtain supernatant which was used as crude cellulase enzyme filtrate for further studies. 0.2 mL of crude cellulase enzyme filtrate was introduced to wells made by a cork borer in CMC agar plates. The plates were soaked with Gram's iodine following incubation for 48 h at room temperature (28 ±2 ˚C). Finally, bacterial isolates with the highest mean diameter of clear zones were selected for further studies (Rawway, Ali & Badawy, 2018).

2.1.2 Cellulase enzyme assays (Secondary screening)

The total cellulase enzyme assay was carried out by placing a rolled filter paper strip (1 × 6 cm) into each test tube and submerged in 0.5 mL of crude enzyme filtrate and 1.0 mL of 50 mM citrate buffer (pH 4.8) as described by Singh *et al*. (2019). Each test tube was incubated at 50˚C for 1 h and the reaction was terminated by adding 3 mL of DNS reagent followed by boiling the mixture for 5 min and adding 20 mL of distilled water. The amount of glucose released as measured using the spectrophotometer (Model: UV-VIS Double UVD 2960) at 540 nm, to obtain the glucose standard curve. Endoglucanase/CMCase was determined by the method described by Singh *et al*. (2019) with a modification of using 0.5 mL of 1% CMC prepared in 50 mM citrate buffer (pH 4.8), instead of filter paper strips. One cellulase unit (U) was defined as the amount of enzyme per milliliter culture filtrate that released 1 µg glucose per minute.

2.2 Isolation of sugar fermenting bacteria

1 mL of fresh palm wine was serially diluted in 0.9% sterile saline solution up to ten-fold (10^{-6}). Thereafter, 1 mL of each dilution was introduced to sterilized petri plates followed by standard pour plate technique (Idroos & Manage, 2018; Liyanage & Manage, 2016a) on YPG agar medium (Glucose, 20 gL^{-1}; Peptone, 5 gL^{-1}; Yeast extract powder, 3 gL^{-1}; $MgSO_4.7H_2O$, 5 gL^{-1}; $CaCl_2$, 1 gL^{-1} and Bacteriological agar, 15 gL^{-1}). All the analytical grade chemicals were purchased from Sigma Aldrich, USA. After 48 h of incubation at 28 ±2˚C, bacterial colonies with different morphological features were selected, isolated and repeatedly streaked on YPG agar plates to obtain pure bacterial cultures following sub-culturing and stored as agar slants at -20˚C in lysogeny agar-glycerol media for further studies (Liyanage & Manage, 2016b). Sugar fermenting bacteria isolated from palm wine were referred to as PW (e.g. PW-7) for reference.

2.2.1 Primary screening of sugar fermenting bacteria

Primary screening for sugar fermentation was based on the Durham tube assay described by Dung & Huynh (2013) and Dung, Thanonkeo and Phong, (2012). The isolated monocultures of bacteria strains were equalized according to 0.5 McFarland standards in 0.9% saline water. 1 mL was inoculated into a test tube containing 5 mL of YPG broth medium (Peptone, 5 gL^{-1}; Yeast extract powder, 3 gL^{-1}; $MgSO_4.7H_2O$, 5 gL^{-1} and $CaCl_2$ 1 gL^{-1}) and the Durham tube was incubated for 48h at room temperature (28 ±2˚C) for gas production.

2.2.2 Secondary screening for sugar fermentation

Multi-dimensional GC-MS (Agilent Technologies, Model: GC system-7890 A, MS system-5975 C) connected to data acquisition software (Chemstation) and HP – 5 ms UI capillary column (5% phenyl methylsiloxane, length - 30 m, thickness – 0.25 µm and diameter 0.25 mm, Agilent) was used for quantitative analysis of ethanol in fermented broth. The general run parameters are: injector, 200˚C; oven temperature program, 40˚C initial, 5 min hold, 10˚C min^{-1}, 43˚C final; carrier gas, GC grade He at 0.5 $mLmin^{-1}$ flow to maintain a pressure of 0.69481 psi, split-less mode. The mass to charge ratio (mz^{-1}) range was set between 35 and 550. Electron ionization energy was set at 70 eV. The MS detector was auto-tuned at the commencement of each day.

2.2.3 SPME (Solid Phase Micro-extraction) conditions

SPME fiber was conditioned before first use by incubation at 250˚C for 20 min. The SPME conditions for GC-MS with an auto-sampler were as follows; about 3 mL aliquot of broth sample was transferred to a 25 mL screw capped vial with polytetrafluoroethylene (PTFE)-lined silicone septa. The vial was agitated for 5 min followed by an overnight incubation at room temperature (28 ±2 ˚C) before SPME extraction. Afterwards, SPME fiber was inserted into the headspace of the vial through the septum of the screw cap. The SPME fiber was exposed in the headspace of the vial for 10 min with each extraction, and then the SPME fiber was removed carefully from the vial and inserted into the GC injection port for analysis.

2.2.4 Quantification of ethanol percentage

Initially, to determine the presence of ethanol and to identify the retention time, mass spectrometry was operated in the scan mode and the total chromatogram of the sample was obtained. Run time was 8 min for the full scan. The transfer line to the MS was maintained at 250˚C and the source temperature was 230˚C while run parameters were followed as described above. Resulted from mass spectrum (MS) graph of ethanol being monitored via Chemstation Software using the ETS09 database and ion fragment 45 mz^{-1} was selected for further analysis of ethanol and quantification was performed operating MS in selected ion monitoring mode (SIM). The standard curve obtained following the injection of the dilutions of standard ethanol solutions (HPLC grade, Sigma Aldrich, USA) ranging 3% - 30% into the GC–MS–SIM instrument and plotting the ion counts (45 mz^{-1}) on the *y*-axis against the percentage of ethanol on the *x*-axis. Finally, the percentages of ethanol in unknown samples were quantified compared to the standard curve of ethanol.

2.3 Optimization of culture conditions for maximum cellulase and ethanol production

The optimization for the medium and process parameters like carbon source, nitrogen source, percentage of phosphorous source, temperature, etc. was carried out with stepwise modifications for the selected cellulase producing and sugar fermenting bacteria isolates for efficient ethanol production.

2.3.1 Maximum cellulase production

Effects of temperature, percentage of CMC, contribution of nitrogen source and percentage of K_2HPO_4 were determined at 28˚C, 33˚C, 37˚C, and 40˚C, following different percentages of CMC (0.20%, 0.40%, 0.80%, 1.20% and 1.60% (w/v)). Effect of nitrogen source was determined supplementing with a series of 0.2% (w/v) nitrogen sources as yeast extract powder, peptone, urea, and ammonium nitrate whereas the effect of phosphorous was determined with varying percentages of K_2HPO_4 (0.05%, 0.10%, 0.15% and 0.20% w/v). To find the effect on enzyme production, cultures were incubated 72 h at 100 rpm. Following which, enzyme activity was assayed by the DNS test for crude cellulase enzyme filtrate.

2.3.2 Maximum sugar fermentation

Effect of ethanol levels, percentage of glucose and temperature were determined by supplemented with different ethanol levels (0, 4, 8 and 12% (v/v)), different percentages of glucose (2, 5, 8, and 12% (w/v)) and temperature at 28, 37, 40 and 50˚C. The sugar fermentation rate was recorded during the incubation time by measuring the gas production in Durham test tubes (Dung, Thanonkeo and Phong, 2012; Dung and Huynh, 2013).

2.4 Characterization and identification of bacterial isolates

Initially, morphological characterizations and biochemical tests were used for the tentative identification of bacteria. Then, bacterial strains were subjected to DNA extraction and shipped to Macrogen (Korea) for the 16S rRNA gene sequence analyses (Idroos & Manage, 2018; Liyanage & Manage, 2016b).

2.5 Production of bioethanol using cellulosic biomass as a substrate (CMC)

2.5.1 Separate Hydrolysis and Fermentation (SHF)

The SHF method was initiated with 1.2% (gmL^{-1}) CMC in 15 mL of CMC broth and 20% of 0.5 McFarland equalized S-6/T-4 strain was inoculated following 72 h incubation at 37˚C for cellulase enzyme production. Then CMC broth was centrifuged at 5000 rpm for 15 min at 4˚C to obtain crude cellulase filtrate. Afterward, hydrolysis was performed at 50˚C for 72 h using crude cellulase enzyme filtrate in a separate unit. The next process, fermentation was conducted after lowering the temperature to 37˚C and 15 mL of glucose-free YPG broth was supplemented following inoculation of 20% of 0.5 McFarland equalized PW-7 strain and incubated for 72 h. The stirring rate was kept at 100 rpm for all the processes and the ethanol percentage was quantified by the SPME method.

2.5.2 Simultaneous Saccharification and Fermentation (SSF)

The SSF method was also initiated with 1.2% (gmL^{-1}) CMC in 15 mL of CMC broth and inoculated 20% of 0.5 McFarland equalized S-6/T-4 strain followed by incubation for 72 h at 37˚C. Then CMC broth was centrifuged at 5000 rpm for 15 min at 4 ˚C to obtain crude cellulase filtrate. Crude cellulase enzyme filtrate was added to 15 mL of glucose-free YPG broth with 20% of 0.5 McFarland equalized PW-7 strain. Afterward, both hydrolysis and fermentation steps were carried out in one unit for 72 h at 37˚C. The stirring rate was kept at 100 rpm for all the processes and the ethanol percentage was quantified by the SPME method. The flow diagram of SHF and SSF process is shown in Fig. 1 (Dahnum *et al.* 2015).

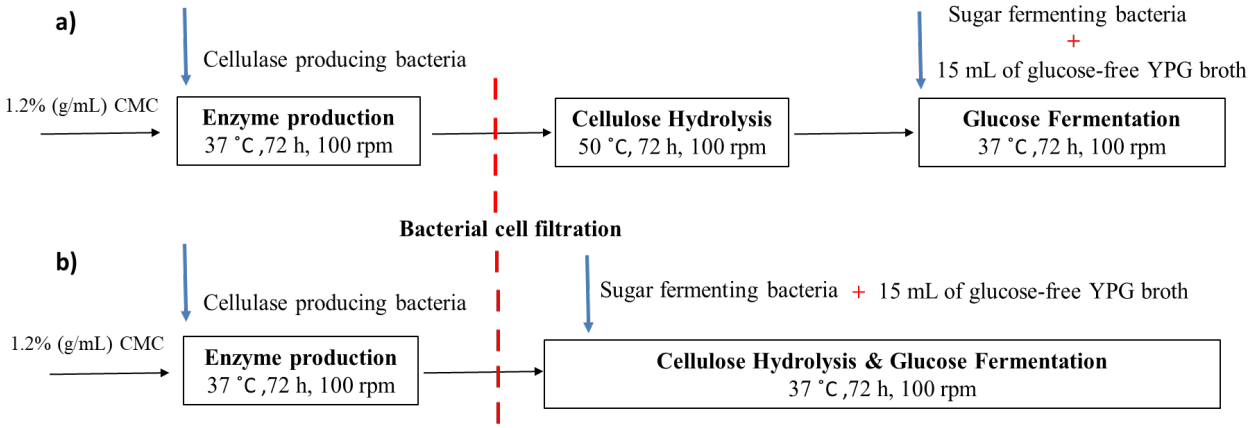

Fig. 1 Experimental set up of different ethanol producing processes (a) separate hydrolysis and fermentation; (b) simultaneous saccharification and fermentation

3. Results and Discussion

Sri Lanka is having the most diverse micro-ecosystem. However, limited research has been recorded on bioethanol production through a biological pathway in Sri Lanka. Therefore, this study was focused to isolate and characterize efficient bacteria for bioethanol production. Animals who feed more cellulose, contain microorganisms in their gut, which are able to produce cellulase enzymes for the degradation of cellulose, were especially sought. Due to the diversity of extreme niche conditions (acid, alkali stability, and thermo-stability) in the gut, cellulase enzymes have the potential to withstand extreme conditions (Tsegaye, Balomajumder & Roy, 2019). In the present study, cow dung, compost soil and termite gut content were selected to isolate the most efficient cellulase-producing bacterial strains. This was the first study regarding the isolation of potential cellulase producing bacteria from the termite gut recorded in Sri Lanka.

3.1 Isolation of bacteria and primary screening for cellulase enzyme

A total of 48 isolates, 27 cellulose-degrading aerobic bacterial strains were isolated from compost soil (25 strains), cow dung (15 strains) and termite gut (08 strains), which were cultured in CMC agar medium following standard pour plate method. Then pure bacterial strains were obtained through repeated streaking of morphologically different bacterial colonies on CMC agar plates as given in Fig. 2.

The cellulase enzyme activity was assessed for isolated pure bacterial cultures by the average diameter (AD) of halo zones in CMC media plates with the presence of Gram's iodine qualitatively. The primary screening of Gram's iodine produces a bluish-black complex with only cellulose (polysaccharide) but not with mono-saccharides such as glucose resulting in clear halo zones. Thus, clear and sharp halo zones indicate cellulase activity (Fig. 3). As showed in Fig. 3, six strains were selected for further studies based on the cellulase enzyme-producing rate (Average Diameter of clear zones (AD) >19.33 ±0.057 mm). Strain T-4, T-2, T-1, S-6, CD-14 and T-5 showed average halo zone diameters as 33.67 ±0.057 mm, 30.67 ±0.06 mm, 30.67 ±0.06 mm, 19.33 ±0.06 mm, 23.33 ±0.06 mm and 28.33 ±0.06 mm respectively.

However, plate screening using dyes is not quantitative due to the poor correlation between enzyme activity and the size of the halo zones (Manzum & Al Mamun, 2018). Thus, cellulase enzyme activity was further assessed with DNS assay.

3.2 Cellulase enzyme assay (Secondary screening)

The filter paper assay (FPA) is the key method for the analysis of total cellulase activity (Sirohi *et al.* 2018). The ability to degrade filter paper represents both production of more than one type of enzyme and the ability to degrade crystalline cellulose. Thus, more efficient cellulase-producing isolates can be identified by the filter paper assay (Mboowa *et al.* 2020). In the present study, only strain S-6 (0.93 ±0.012 U) and strain T-4 (0.35 ±0.021 U) showed the total cellulase activity as represented in Fig. 4. Endoglucanase (CMCase) is an enzyme that cleaves intermolecular ß-1,4-glucosidic bonds on the surface of cellulose randomly (Zou *et al.* 2018) and the results of the present study showed that the strain S-6 (1.56 ±0.021 U) having the highest endoglucanase activity while second highest was T-4 resulting 0.21 ±0.021 U activity which was similar with the results recorded by Ladeira *et al.* (2015).

3.3 Isolation of bacteria & primary screening for ethanol production

In the study, the fermenting capacity of isolates was assessed through Durham tube assay by measuring gas production (Dung & Huynh, 2013; Dung, Thanonkeo & Phong, 2012). It has been recorded that the volume of gas produced, was related to the fermenting rate of the fermenting bacterial strain. Results of the present study revealed that out of 8 isolates only one strain (PW-7) as possessing the sugar fermenting ability as given in Fig. 5. Within 48 h it showed the maximum height of CO_2 in Durham tube which was 40 mm (Arrow shows the gas trapped Durham tube). Thus, PW-7 strain was selected for further studies.

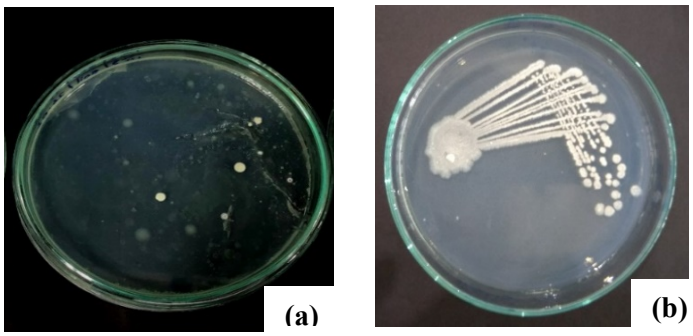

Fig. 2 Isolation of cellulase producing bacteria (S-6) from compost soil; (a) Pour plate; (b) Streak plate

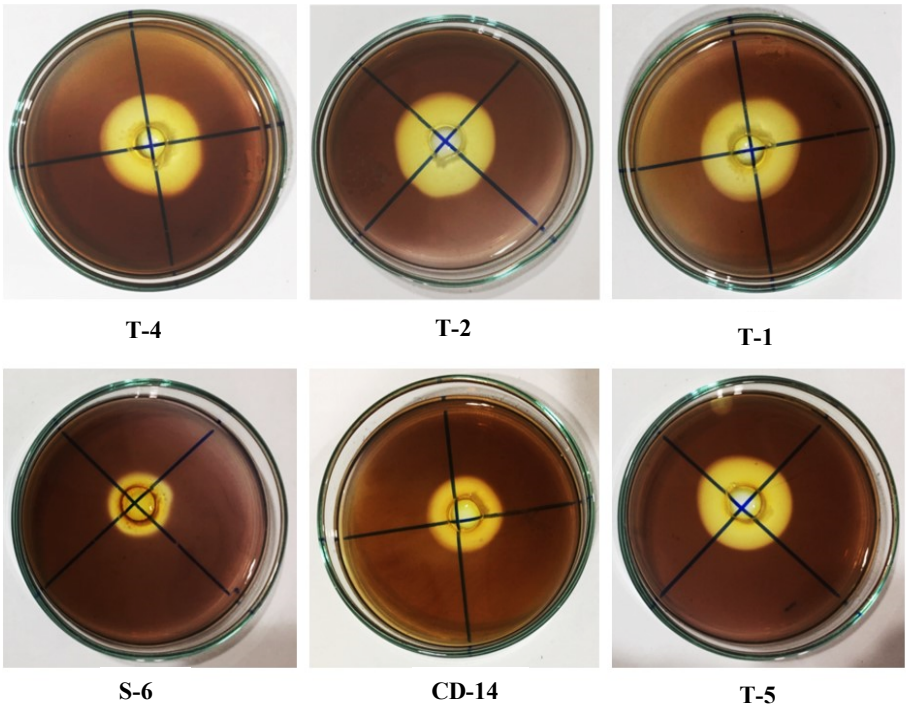

Fig. 3 Clear zones with Gram's iodine on Carboxy Methyl Cellulose (CMC) medium for the selected bacteria

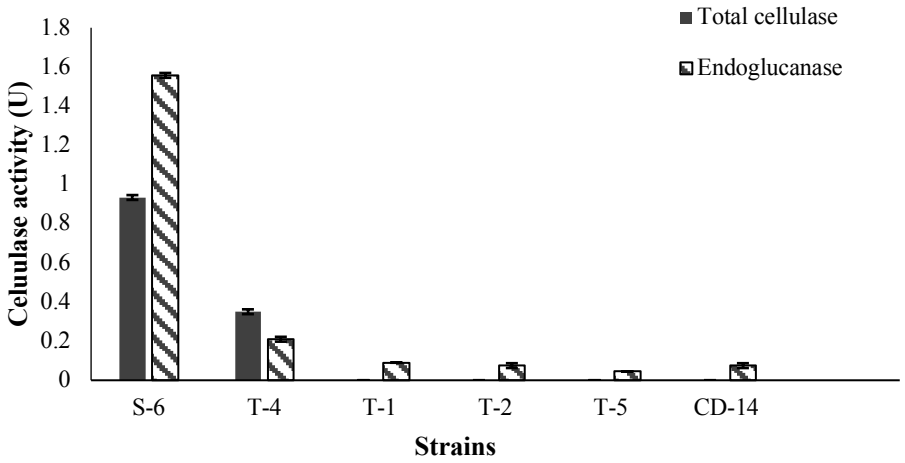

Fig. 4 Total cellulase and endoglucanase activity at 50 ˚C with crude enzyme filtrates after 3 days of incubation

3.4 Secondary screening for ethanol production

Quantitative analysis of ethanol percentage was carried out by SPME coupled with GC-MS, which can be used as an alternative method to overcome some practical problems such as extensive sample preparation, cost intensive equipment requirements, use of solvents and complicated procedures causing handling errors, etc. SPME is a simple, solvent-free technique that enables the quantification of a high number of compounds with low concentrations (Ansari & Karimi, 2017). However, there are some critical parameters for volatile sample preparation by SPME in GC-MS which are: sample temperature, sample volume, extraction time, and solution matrix (Rebière *et al.* 2010). As cited in Onuki *et al.* (2016) all compounds showed high linearity in the extraction time ranging from 10 to 20 min for ethanol. Relatively short extraction time minimizes the risk of compound displacement from fiber coating, competitive adsorption among target compounds, and also minimized the negative effects of limited SPME fiber sorbent capacity. In the present study, all the parameters for each sample were maintained in the same conditions as 3 mL sample volume, overnight incubation at room temperature (28 ±2˚C) and 10 min exposure time. The total scan chromatogram was showed two retention time values for ethanol as 2.158 and 2.286 (Fig. 6).

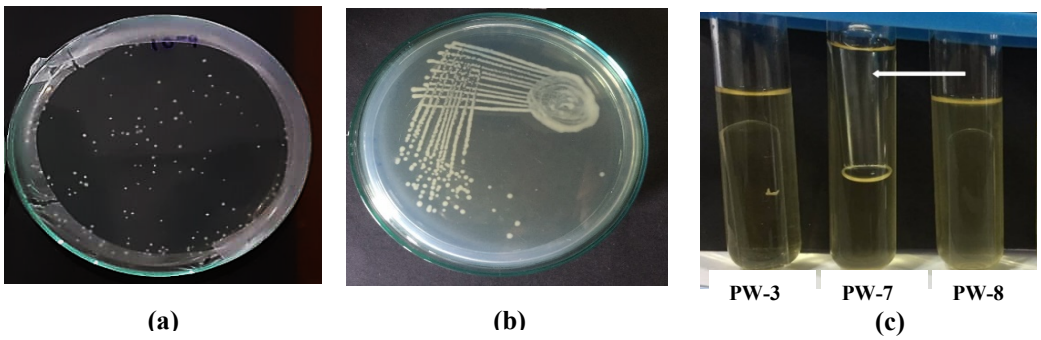

Fig. 5 Isolation and primary screening of sugar fermenting bacteria from palm wine

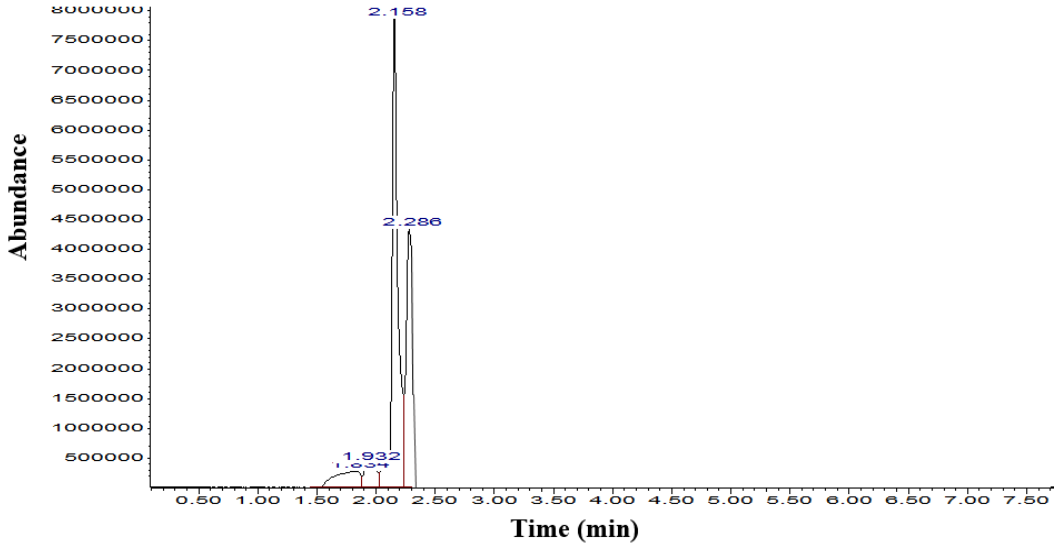

Fig. 6 Total scan chromatogram for fermented broth for 8 min

Thus, the mass spectrum (MS) of ethanol monitored via Chemstation Software using the ETS09 database. 31 and 45 mz^{-1} ion fragments resulted as the most abundant fragments for ethanol (Fig. 7). For further analysis of ethanol, 45 mz^{-1} ion fragment was selected which was closer to the molar mass of ethanol (46.07 gmol-1). The ethanol standard curve obtained by the plotting percentage of ethanol (v/v) versus ion count showed excellent linearity with the correlation coefficient (R^2 = 0.9923). Therefore, a strong relationship was found between ethanol percentages against ion count. Thus, the slope of the standard curve was used to obtain the percentage of ethanol in samples fermented samples.

Generally, at the end of the fermentation, only about 0.5 – 5% of bioethanol was contained in the broth medium (Torres & Rong, 2016). The results of the present study revealed that strain PW-7 has the potential to produce 3.07% of ethanol content in broth medium according to SIM mode analysis.

3.5 Characterization and identification of bacteria

Observing different morphological features, 48 strains were isolated from cow dung (15 strains), compost soil (25 strains) and termite gut content (08 strains). Out of these, 27 showed cellulase activity and by the primary screening, 6 strains were selected for further studies following the results of secondary screening revealed that only 2 bacteria strains had the potential for cellulase production. They were identified as *Nocardiopsis* sp. (S-6) and *Bacillus* sp. (T-4) by 16S rRNA gene sequence analysis.

For sugar fermentation, eight morphologically different bacterial strains were isolated from palm wine. However, performing primary screening through Durham tube assay revealed that only 1 strain had the intrinsic potential for sugar fermentation and this strain was identified as *Achromobacter* sp. (PW-7) by 16S rRNA gene sequence analysis.

3.6 Optimization of culture conditions for maximum cellulase and ethanol production

3.6.1 Maximum cellulase production

Cellulase is an inducible enzyme through medium optimization since there is a complex relationship between bacterial growth and enzyme yields with a variety of environmental factors such as pH value, temperature, medium additives, growth time, and so forth (Sharma *et al.* 2019; Weerasinghe, Madusanka & Pathmalal, 2020). Thus, optimization is a cost-effective and important aspect to obtain the maximum yields of cellulase. Temperature plays a significant role in the physiology and growth of

microorganisms and also their enzyme activities. At their optimum temperature, enzymes work rapidly and many enzymes become partly unfolded and inactivated at elevated temperatures (Tacias-Pascacio *et al.* 2019). As cited in Islam & Roy, (2018) generally *Bacillus* sp. produce less thermostable cellulase. In the present study both *Bacillus* sp. and *Nocardiopsis* sp. showed the maximum cellulase production at 37˚C and similar results were recorded by Lugani, Singla & Sooch (2015) and Tabssum *et al.* (2018).

Carbon is the basic form of nutrients required for bacterial growth. Carboxy methyl cellulose (CMC) was found to support maximum cellulase yield due to its less complexity and easy digestion by the microbes (El-Sayed *et al.* 2019). Thus, in the present study, CMC was supplemented as the sole carbon source and the percentage of CMC was optimized and the highest cellulase yield was obtained (1.90 ±0.042 U) for S-6 with 1.2% CMC concentration whereas T-4 showed greater results with 1.6% CMC concentration (Fig. 8b). Islam & Roy, (2018) had recorded similar results showing that the maximum enzyme activity was achieved from *Bacillus* sp. with the presence of 1% CMC as the carbon source.

The nutrients such as nitrate and phosphorus have a strong influence on the microorganism community and activity (Ekanayake & Manage, 2016). The addition of peptone as a nitrogen source enhanced the cellulase enzyme yield other than Yeast Extract Powder, NH_4NO_3 and Urea were recorded in the study. There was no bacterial growth observed with urea and only *Bacillus* sp. (T-4) showed a lower cellulase activity (0.10 ±0.042 U) when NH_4NO_3 was employed as the sole nitrogen source. This could be probably due to the metabolism of inorganic nitrogen leads to medium acidification and it could be in turn negatively effects on cellulase production. Maravi & Kumar, (2021) supported the results of the present study on the utilization of NH_4NO_3 or urea as sole nitrogen sources. Many studies have shown K_2HPO_4 as the phosphorous source and no records regarding optimization of K_2HPO_4 percentage for maximum enzyme production. The present study yielded high cellulase activity (1.89 U) when medium was modified with 0.20% K_2HPO_4 (Fig. 8d).

3.6.2 Maximum ethanol production

Final ethanol concentration in broth media is significant and the productivity of ethanol is affected by many factors including medium composition, pH, temperature, product inhibition and also the choice of microorganisms. Ethanol is an inhibitor of microbial growth even at relatively low concentrations, inhibiting cell division, decreasing cell volume and specific growth rate while in high ethanol concentrations, reduce cell vitality and increase cell death (Saeed *et al.* 2018). The results showed that the selected bacteria strains were able to tolerate percentage in the medium up to 12% and the fermentation potential (Height of CO_2 in Durham tubes) decreased with the increase of ethanol percentage (4%, 8% and 12%) in fermentation medium (Fig. 9a). The rapid growth of microbes will be decreased with limited nutrients. However, higher initial glucose concentrations in the fermentative media may cause to substrate inhibition, which lowers the fermentation efficiency. Thus, to improve the fermentation process, the optimization of the glucose concentration in cultural media is necessary. According to the results of the present study, the highest ethanol production was performed (40 mm) within 12 h incubation with 8% and 12% glucose percentages, while ethanol production was maximized with 2% and 5% glucose percentages following 36 h and 24 h of incubation respectively (Fig. 9 b). The sugar fermentation capacity of *Achromobacter* sp. was evaluated at different temperatures and results revealed that *Achromobacter* sp. could grow well and ferment sugar at 28˚C and 40˚C while 37˚C was recorded as having the highest gas discharge rate. However, there was no gas production and bacterial growth at 50˚C even after 48 h incubation period (Fig. 9c).

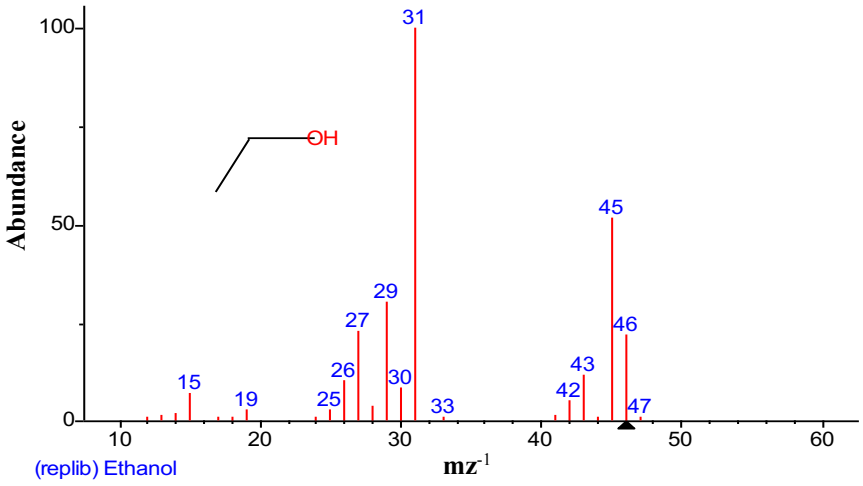

Fig. 7 Scan mass spectrum of ethanol monitored via Chemstation Software using ETS09 database

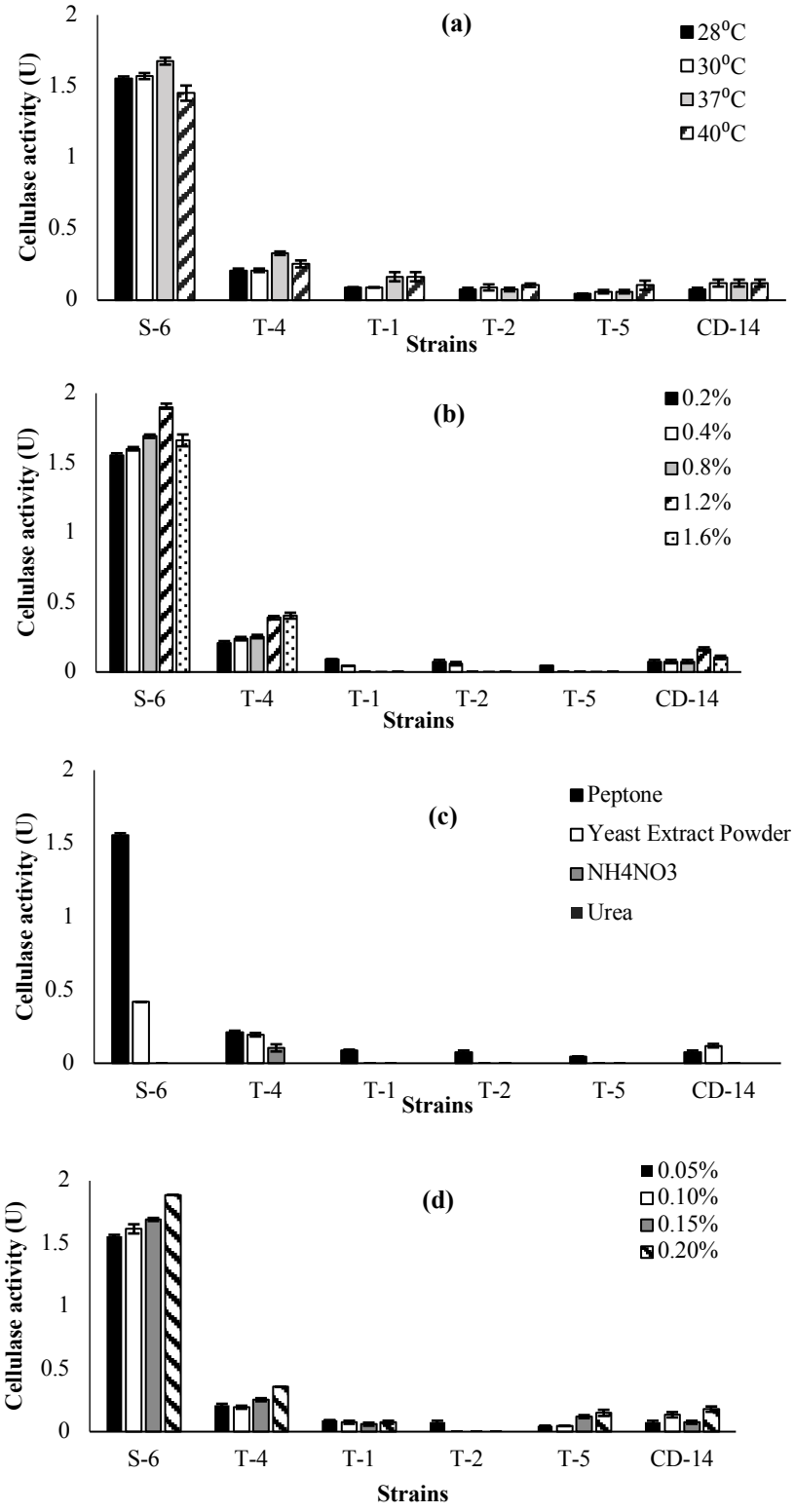

Fig. 8 Effect of different parameters on cellulase production (a) Temperature; (b) Percentage of Carboxy Methyl Cellulose (CMC); (c) Nitrogen Source; (d) Percentage of K_2HPO_4 (Phosphorus source)

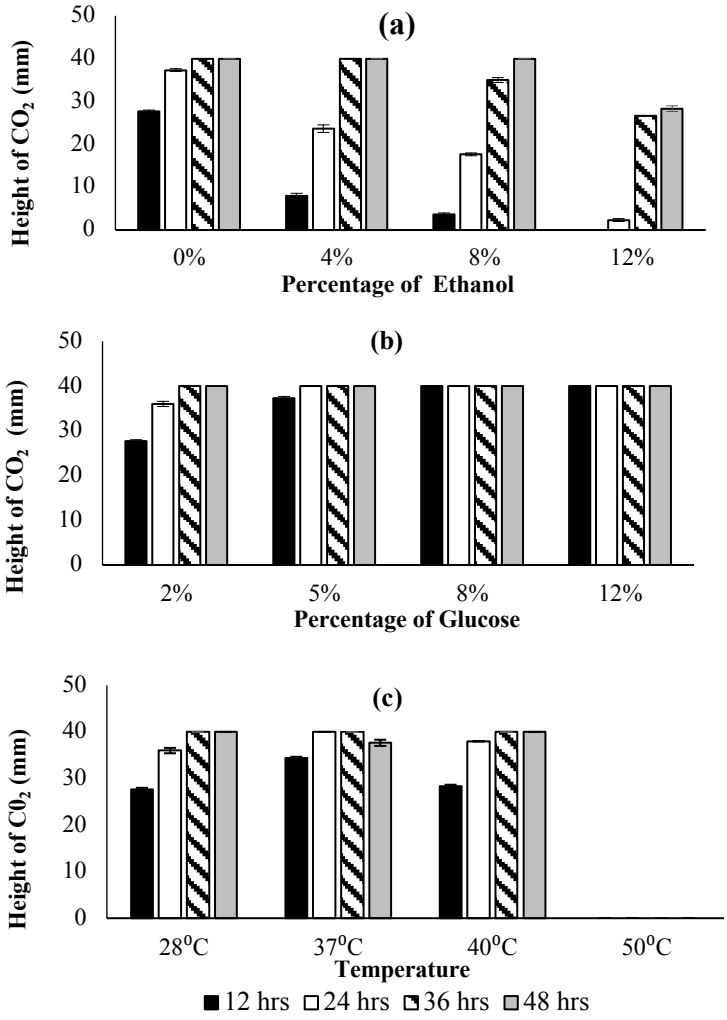

Fig. 9 Effect of different parameters on ethanol production (a) Percentage of ethanol level; (b) Percentage of glucose; (c) The incubation temperature (No results were found for temperature at 50 ˚C)

3.7 Integration of cellulose hydrolysis and sugar fermentation

This study revealed that though *Nocardiopsis* sp. has the highest enzyme activity, simultaneous saccharification and fermentation (SSF) produced a higher amount of ethanol (1.67%) when *Bacillus* sp. and *Achromobacter* sp. were employed (Table 1). As recorded in Muthukrishnan *et al.* (2020), the genus *Nocardiopsis* has the greatest potential as a source of antimicrobial agents. Thus, secretions of *Nocardiopsis* sp. could lead to inhibitory effects on *Achromobacter* sp. activity in the sugar fermentation process resulting in a much lower amount of ethanol as given in Table 1.

As cited by Pandey *et al.* (2019), the simultaneous saccharification and fermentation (SSF) process require a low concentration of cellulase enzyme and a higher amount of ethanol can be obtained than from SHF. This is due to its ability for rapid conversion of sugars which was inhibitory for the saccharification process into ethanol as soon as they are formed without accumulation in the medium and also the degree of contamination is lowering (Saleem *et al.* 2020). Thus, cellulose hydrolysis and sugar fermentation through simultaneous saccharification and fermentation (SSF) was the most efficient method compared to separate hydrolysis and fermentation (SHF) for bioethanol production.

The interest for cellulase enzyme has augmented with the exploration of greener, environment-friendly alternative fuels such as bioethanol. The present study records mesophilic bacteria for cellulase production and usually they generate low yields of sugars from lignocellulose due to incomplete and slow enzymatic hydrolysis rates and are prone to microbial contamination problems. It has been suggested that such limitations could be overcome by using thermophilic bacteria and thermostable enzymes. However, the application of thermophilic microbes in industrial settings of bioethanol production is costly due to high maintenance cost. Further, the low concentrations of crude cellulase enzyme in broth would also be a disadvantage. Therefore, further improvements are recommended to enhance the enzyme activity through cellulase enzyme purification process. For industrial bioethanol production, biomass should be cheap and easily available in order to achieve the economic targets required to replace fossil fuels. Currently, the recycling of paper waste is considerably low, a value of 20-25% of the total paper waste in Sri Lanka.

Table 1
Percentage of ethanol in fermented broth samples (*Achromobacter* sp.; sugar fermenting bacteria, *Nocardiopsis* sp. and *Bacillus* sp.; cellulose hydrolyzing bacteria)

Condition	Strains	% of ethanol
Separate enzymatic hydrolysis & fermentation (SHF)	*Nocardiopsis* sp. + *Achromobacter* sp.	1.28%
	Bacillus sp. + *Achromobacter* sp.	1.25%
Simultaneous saccharification and fermentation (SSF)	*Nocardiopsis* sp. + *Achromobacter* sp.	1.05%
	Bacillus sp. + *Achromobacter* sp.	1.67%

Paper waste seems the most suitable feedstock for ethanol production as it contains a higher amount of cellulose and a lower amount of lignin and hemicellulose. Thus, further studies should be carried out to accommodate paper waste as biomass for ethanol production. Ethanol can be blend with petro-diesel and the ethanol-diesel blend is a successful alternative for pure diesel. According to many literatures, E5 (Ethanol 5% with 95% petro-diesel) as a lower blend, can be used for diesel engines without any modifications. Even with a lower blend such as E5, the depletion of petro-diesel can be reduced by 5% and it can be extended by 5% intervals. Thus, further studies are being conducted to assess the acceptability of ethanol-biodiesel blends and to mitigate deficiencies that are associated with the higher ethanol-diesel blends without any engine modifications. Thus, the current study will be addressed energy crisis to future as a green solution while supporting paper waste management.

4. Conclusion

The results of the present study revealed that out of *Nocardiopsis* sp. and *Bacillus* sp., *Nocardiopsis* sp. has a higher potential to produce cellulase enzyme and *Achromobacter* sp. converts glucose into ethanol with high efficiency. Both *Nocardiopsis* sp. and *Bacillus* sp. showed the same optimum conditions for maximum cellulase production; peptone as a nitrogen source, 0.2% K_2HPO_4 as phosphorus source, 37 ±2˚C temperature, with a 1.2% CMC concentration. *Nocardiopsis* sp. and *Bacillus* sp. showed greater results with 1.6% CMC concentration, 8% glucose, and 37 ±2˚C temperature as optimal conditions for ethanol production Simultaneous saccharification and fermentation (SSF) was found to be the most efficient bioprocess for bioethanol production. Production of ethanol from cellulosic biomass is technically-feasible and further studies are mandatory for diesel-ethanol blends, before utility as an alternative for petro-diesel as it has to comply with ASTM or EU standards.

References

Aditiya, H.B., Mahlia, T.M.I., Chong, W.T., Nur, H. and Sebayang, A.H., (2016). Second generation bioethanol production: A critical review. *Renewable and sustainable energy reviews,* 66, 631-653; doi.org/10.1016/j.rser.2016.07.015

Ahmadi, P., Dincer, I. and Rosen, M.A., (2015). Performance assessment of a novel solar and ocean thermal energy conversion based multigeneration system for coastal areas. *Journal of Solar Energy Engineering*, 137(1); doi.org/10.1115/1.4028241

Ansari, S. and Karimi, M., (2017). Recent progress, challenges and trends in trace determination of drug analysis using molecularly imprinted solid-phase microextraction technology. *Talanta*, 164, 612-625; doi.org/ 10.1016/j.talanta.2016.11.007

Anu, Kumar, S., Kumar, A., Kumar, V. and Singh, B., (2020). Optimization of cellulase production by Bacillus subtilis subsp. subtilis JJBS300 and biocatalytic potential in saccharification of alkaline-pretreated rice straw. *Preparative Biochemistry & Biotechnology,* 1-8; doi.org/ 10.1080/10826068.2020.1852419

Azhar, S.H.M., Abdulla, R., Jambo, S.A., Marbawi, H., Gansau, J.A., Faik, A.A.M. and Rodrigues, K.F., (2017). Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports,* 10, 52-61; doi.org/10.1016/j.bbrep.2017.03.003

Balkhair, K.S. and Rahman, K.U., (2017). Sustainable and economical small-scale and low-head hydropower generation: A promising alternative potential solution for energy generation at local and regional scale. *Applied Energy*, 188, 378-391; doi.org/10.1016/j.apenergy.2016.12.012

Dahnum, D., Tasum, S.O., Triwahyuni, E., Nurdin, M. and Abimanyu, H., (2015). Comparison of SHF and SSF processes using enzyme and dry yeast for optimization of bioethanol production from empty fruit bunch. *Energy Procedia*, 68, 107-116; doi.org/10.1016/j.egypro.2015.03.238

Dung, N.T.P. and Huynh, P.X., (2013). Screening Thermo-and Ethanol Tolerant Bacteria for Ethanol Fermentation. *American Journal of Microbiological Research,* 1(2), 25-31; doi.org/10.12691/ajmr-1-2-3

Dung, N.T.P., Thanonkeo, P. and Phong, H.X., (2012). Screening useful isolated yeasts for ethanol fermentation at high temperature. *International Journal of Applied*, 2(4), 65-71

Ekanayake, E.M.M.S. and Manage, P.M., (2020). Green approach for decolorization and detoxification of textile dye-CI direct blue 201 using native bacterial strains. *Environment and Natural Resources Journal,* 18(1),.1-8

Ekanayake, M.S. and Manage, P.M., (2016). Isolation of Textile Dye Decolorizing Bacteria from Environmental Samples. Symposium Proceedings, Fifth International Symposium on Water Quality and Human Health: Challenges Ahead, 05 & 06 August, PGIS, Peradeniya, Sri Lanka

El-Sayed, A.F., Abo-Sereih, N.A., Mahmoud, A.E., El-Kawokgy, T.M. and El-Ghamery, A.A., (2019). Genetic identification and optimization of novel ß-glucosidase-producing Lysinibacillus sphaericus QS6 strain isolated from the Egyptian environment. *Egyptian Pharmaceutical Journal*, 18(4), 341; doi.org/10.4103/epj.epj_51_19.

Gunawardena.S., (2009). Liquid biofuel for transportation in srilanka., Technical report, University of Moratuwa

Hassan, S.S., Williams, G.A. and Jaiswal, A.K., (2019). Moving towards the second generation of lignocellulosic biorefineries in the EU: Drivers, challenges, and opportunities. *Renewable and Sustainable Energy Reviews,* 101, 590-599; doi.org/10.1016/j.rser.2018.11.041

Idroos, F.S. and Manage, P.M., (2018). Bioremediation of Microcystins by Two Native Bacteria; Bacillus Cereus and

Rahnella Aquatilis. *Asian Journal of Microbiology Biotechnology and Environmental Sciences*, 20(3), 24-32

Islam, F. and Roy, N., (2018). Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. *BMC research notes*, 11(1),1-6; doi.org/10.1186/s13104-018-3558-4

Jayasekara, S.K., Abayasekara, C.L. and Ratnayake, R.R., (2019). Efficient Microorganisms for Bioethanol Production from the Natural Environment of Sri Lanka. In Proceedings of International Research Symposium of Uva Wellassa University. doi.org/10.13140/RG.2.2.13363.25120

Jayathilaka, M.G.L.W., Henagamage, A.P., Peries, C.M. and Seneviratne, G., (2018). Enhancement of Cellulolytic Activity through Biofilm Action for Bioethanol Production. In Proceedings of International Research Symposium of Uva Wellassa University

Kularathne, I.W., Rathneweera, A.C., Kalpage, C.S., Rajapakshe, S. and Gunathilaka, C.A., (2020). Optimization and kinetic parameter estimation of bioethanol production from freely available Sri Lankan fruits in batch fermentation. Ceylon *Journal of Science*, 49(3), 283-291; doi.org/10.4038/cjs.v49i3.7779

Ladeira, S.A., Cruz, E., Delatorre, A.B., Barbosa, J.B. and Leal Martins, M.L., (2015). Cellulase production by thermophilic Bacillus sp: SMIA-2 and its detergent compatibility. *Electronic journal of biotechnology*, 18(2), 110-115; doi.org/10.1016/j.ejbt.2014.12.008

Liu, C.G., Xiao, Y., Xia, X.X., Zhao, X.Q., Peng, L., Srinophakun, P. and Bai, F.W., (2019). Cellulosic ethanol production: progress, challenges and strategies for solutions. *Biotechnology advances*, 37(3), 491-504; doi.org/10.1016/j.biotechadv.2019.03.002

Liyanage, G.Y and Manage, P.M., (2016a). Isolation and characterization of oil degrading bacteria from coastal waters and sediments from three locations in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka,* 44(4), 201-203; doi.org/10.4038/jnsfsr.v44i4.8017

Liyanage, G.Y and Manage, P.M., (2016b). Optimization of Environmental Factors on Oil Degrading Bacteria Isolated from Coastal Water and Sediments in Sri Lanka. *Journal of Tropical Forestry and Environment,* 5(2), 13-25; doi.org/10.31357/jtfe.v5i2.2655

Lu, M., Li, J., Han, L. and Xiao, W., (2019). An aggregated understanding of cellulase adsorption and hydrolysis for ball-milled cellulose. *Bioresource technology*, 273, 1-7; doi.org/ 10.1016/j.biortech.2018.10.037

Lugani, Y., Singla, R. and Sooch, B.S., (2015). Optimization of cellulase production from newly isolated Bacillus sp. Y3. *Journal of Bioprocessing & Biotechniques*, 5(11), 1; doi.org/10.4172/2155-9821.1000264

Madusanka D. A. T. and Manage P. M., (2018). Optimizing a solvent system for lipid extraction from cyanobacterium Microcystis spp.: Future perspective for biodiesel production. *Journal of National Sciences Foundation*, 46(2), 219-227; doi.org/10.4038/jnsfsr.v46i2.8422

Magocha, T.A., Zabed, H., Yang, M., Yun, J., Zhang, H. and Qi, X., (2018). Improvement of industrially important microbial strains by genome shuffling: Current status and future prospects. *Bioresource technology*, 257, 281-289; doi.org/ 10.1016/j.biortech.2018.02.118

Manzum, A.A. and Al Mamun, M.A., (2018). Isolation of Bacillus spp. bacteria from soil for production of cellulase. Nepal *Journal of Biotechnology*, 6(1), 57-61; doi.org/ 10.3126/njb.v6i1.22338

Maravi, P. and Kumar, A., (2021). Optimization and statistical modeling of microbial cellulase production using submerged culture. *Journal of Applied Biology & Biotechnology*, 9(2), 142-152; doi.org/ 10.7324/JABB.2021.9213

Mboowa, D., Chandra, R.P., Hu, J. and Saddler, J.N., (2020). Substrate characteristics that influence the Filter paper assay's ability to predict the hydrolytic potential of cellulase mixtures. ACS Sustainable Chemistry & Engineering, 8(28), 10521-10528; doi.org/10.1021/acssuschemeng.0c02883

Mohanty, S.K. and Swain, M.R., (2019). Bioethanol production from corn and wheat: food, fuel, and future. In Bioethanol production from food crops, 45-59; doi.org/10.1016/B978-0-12-813766-6.00003-5

Nair, R.B. and Taherzadeh, M.J., (2016). Valorization of sugar-to-ethanol process waste vinasse: a novel biorefinery approach using edible ascomycetes filamentous fungi. *Bioresource technology,* 221, 469-476; doi.org/10.1016/j.biortech.2016.09.074

Niegel, J., (2018). Tidal Waves. *Trusts & Trustees*, 24(6), 463-473; doi.org/10.1093/tandt/tty091

Onuki, S., Koziel, J.A., Jenks, W.S., Cai, L., Rice, S. and van Leeuwen, J., (2016). Optimization of extraction parameters for quantification of fermentation volatile by-products in industrial ethanol with solid-phase microextraction and gas chromatography. *Journal of the Institute of Brewing*, 122(1), 102-109; doi.org/10.1002/jib.297

Pandey, A.K., Kumar, M., Kumari, S., Kumari, P., Yusuf, F., Jakeer, S., Naz, S., Chandna, P., Bhatnagar, I. and Gaur, N.A., (2019). Evaluation of divergent yeast genera for fermentation-associated stresses and identification of a robust sugarcane distillery waste isolate Saccharomyces cerevisiae NGY10 for lignocellulosic ethanol production in SHF and SSF. *Biotechnology for biofuels,* 12(1), 1-23; doi.org/ 10.1186/s13068-019-1379-x

Phuog, N.T., Lena, N.T. and Thang, N.D., (2015). Isolation of cellulose degrading bacteria from the gut of the termite Coptotermes gestroi. *Global Science Publications*, 17(4), 931-936

Pramanik, S.K., Mahmud, S., Paul, G.K., Jabin, T., Naher, K., Uddin, M.S., Zaman, S. and Saleh, M.A., (2021). Fermentation optimization of cellulase production from sugarcane bagasse by Bacillus pseudomycoides and molecular modeling study of cellulase. *Current Research in Microbial Sciences*, 2,100013; doi.org/10.1016/j.crmicr.2020.100013

Rajwade, J.M., Paknikar, K.M. and Kumbhar, J.V., (2015). Applications of bacterial cellulose and its composites in biomedicine. *Applied microbiology and biotechnology*, 99(6), 2491-2511; doi.org/10.1007/s00253-015-6426-3

Rastogi, M. and Shrivastava, S., (2017). Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. *Renewable and Sustainable Energy Reviews*, 80, 330-340; doi.org/ 10.1016/j.rser.2017.05.225

Rawway, M., Ali, S.G. and Badawy, A.S., (2018). Isolation and identification of cellulose degrading bacteria from different sources at Assiut governorate (Upper Egypt). *J. Ecol. Heal. Environ. Int. J,* 6, 15-24; doi.org/ 10.18576/jehe/060103

Rebière, L., Clark, A. C., Schmidtke, L. M., Prenzler, P. D., & Scollary, G. R. (2010). A Robust Method for Quantification of Volatile Compounds within and between Vintages Using Headspace-Solid-Phase Micro-Extraction Coupled with GC–MS – Application on Semillon Wines. *Analytica Chimica Acta*, 660(1–2), 149-157; doi.org/10.1016/j.aca.2009.10.029

Saeed, M.A., Ma, H., Yue, S., Wang, Q. and Tu, M., (2018). Concise review on ethanol production from food waste: development and sustainability. *Environmental Science and Pollution Research,* 25(29), 28851-28863; doi.org/10.1007/s11356-018-2972-4

Saleem, A., Hussain, A., Chaudhary, A., Iqtedar, M., Javid, A. and Akram, A.M., (2020). Acid hydrolysis optimization of pomegranate peels waste using response surface methodology for ethanol production. *Biomass Conversion and Biorefinery*, 1-12; doi.org/ 10.1007/s13399-020-01117-x

Sarkar, D., Prajapati, S., Poddar, K. and Sarkar, A., (2020). Ethanol production by Klebsiella sp. SWET4 using banana peel as feasible substrate. *Biomass Conversion and Biorefinery,* 1-13; doi.org/10.1007/s13399-020-00880-1

Sarsaiya, S., Awasthi, S.K., Awasthi, M.K., Awasthi, A.K., Mishra, S. and Chen, J., (2018). The dynamic of cellulase

activity of fungi inhabiting organic municipal solid waste. *Bioresource technology*, 251, 411-415; doi.org/10.1016/j.biortech.2017.12.011

Senarathna, D.B.G.M., Rupasinghe, C.P. and Bandara, W.B.M.A.C., (2019). Bioethanol Production from Lignocellulosic Materials. In Proceedings of EdHat International Research Conference on Technology and Innovation (IRCTECiN)

Sharma, A., Choudhary, J., Singh, S., Singh, B., Kuhad, R.C., Kumar, A. and Nain, L., (2019). Cellulose as potential feedstock for cellulase enzyme production: versatility and properties of various cellulosic biomasses. In New and Future Developments in Microbial Biotechnology and Bioengineering, 11-27; doi.org/10.1016/B978-0-444-64223-3.00002-3

Singh, S., Jaiswal, D.K., Sivakumar, N. and Verma, J.P., (2019). Developing efficient thermophilic cellulose degrading consortium for glucose production from different agro-residues. *Frontiers in Energy Research*, 7, 61; doi.org/10.3389/fenrg.2019.00061

Sirohi, R., Singh, A., Tarafdar, A. and Shahi, N.C., (2018). Application of genetic algorithm in modelling and optimization of cellulase production. *Bioresource technology,* 270, 751-754; doi.org/10.1016/j.biortech.2018.09.105

Song, B., Li, B., Wang, X., Shen, W., Park, S., Collings, C., Feng, A., Smith, S.J., Walton, J.D. and Ding, S.Y., (2018). Real-time imaging reveals that lytic polysaccharide monooxygenase promotes cellulase activity by increasing cellulose accessibility. *Biotechnology for biofuels*, 11(1), 1-11; doi.org/10.1186/s13068-018-1023-1

Szilvia, D., Antalné, T., Tamás, E. and Horská, E., (2015). The Change of Residents' Attitudes Towards Renewable Energy Sources In 2006–2014 As A Reflection Of Primary Research. *Visegrad Journal on Bioeconomy and Sustainable Development*, 4(1), 17-21; doi.org/10.1515/vjbsd-2015-0004

Tacias-Pascacio, V.G., García-Parra, E., Vela-Gutiérrez, G., Virgen-Ortiz, J.J., Berenguer-Murcia, Á., Alcántara, A.R. and Fernandez-Lafuente, R., (2019). Genipin as an emergent tool in the design of biocatalysts: mechanism of reaction and applications. *Catalysts,* 9(12), 1035; doi.org/10.3390/catal9121035

Tabssum, F., Irfan, M., Shakir, H.A. and Qazi, J.I., (2018). RSM based optimization of nutritional conditions for cellulase mediated Saccharification by Bacillus cereus. *Journal of biological engineering*, 12,1-10; doi.org/ 10.1186/s13036-018-0097-4

Thakshika, G., Peries, C.M. and Henegamage, A.P., (2019). Development of Bioethanol from Water Hyacinth (Eichhornia crassipes) Using Cellulose Degrading Microbial Biofilm. In Proceedings of International Research Symposium of Uva Wellassa University

Tsegaye, B., Balomajumder, C. and Roy, P., (2019). Isolation and characterization of novel lignolytic, cellulolytic, and hemicellulolytic bacteria from wood-feeding termite Cryptotermes brevis. *International Microbiology*, 22(1), 29-39; doi.org/ 10.1007/s10123-018-0024-z

Torres-Ortega, C.E. and Rong, B.G., (2016). Intensified separation processes for the recovery and dehydration of bioethanol from an actual lignocellulosic fermentation broth. *In Computer Aided Chemical Engineering*, 38, 727-732; doi.org/10.1016/B978-0-444-63428-3.50126-0

Weerasinghe, W.M.L.I., Madusanka, D.A.T. and Pathmalal, M.M., (2019), November. Isolation of Cellulase Producing Bacteria: Future Perspective for Bio-Ethanol Production. In Proceedings of International Forestry and Environment Symposium. 24; doi.org/10.31357/fesympo.v24i0.4336.g3443

Weerasinghe W.M.L.I., Madusanka D.A.T. and Pathmalal M.M., (2020), December. Isolation and Optimization of cellulase producing bacteria for Second-generation bio-ethanol production. In Proceedings of International Conference on Multi-Disciplinary Approaches (iCMA). 116

Westman, J.O. and Franzén, C.J., (2015). Current progress in high cell density yeast bioprocesses for bioethanol production. *Biotechnology journal*, 10(8), 1185-1195; doi.org/10.1002/biot.201400581

World Bank. (2017). Sri Lanka Development Update, June 2017. Washington, DC. © World Bank [online]. Available at: https://openknowledge.worldbank.org/handle/10986/27519License: CC BY 3.0 IGO [October 2019]

World bioenergy association. (2018). WBA Global Bioenergy Statistics 2018 Summary Report [online]. Available at: https://worldbioenergy.org/uploads/181017%20WBA%20GBS%202018_Summary_hq.pdf [December 2019]

Yadav, S.K., (2017). Technological advances and applications of hydrolytic enzymes for valorization of lignocellulosic biomass. *Bioresource technology*, 245, 1727-1739; doi.org/ 10.1016/j.biortech.2017.05.066

Zeng, Z., Ziegler, A.D., Searchinger, T., Yang, L., Chen, A., Ju, K., Piao, S., Li, L.Z., Ciais, P., Chen, D. and Liu, J., (2019). A reversal in global terrestrial stilling and its implications for wind energy production. *Nature Climate Change*, 9(12), 979-985; doi.org/10.1038/s41558-019-0622-6

Zhao, X., Xiong, L., Zhang, M. and Bai, F., (2016). Towards efficient bioethanol production from agricultural and forestry residues: exploration of unique natural microorganisms in combination with advanced strain engineering. *Bioresource technology*, 215, 84-91; doi.org/10.1016/j.biortech.2016.03.158

Zou, G., Jiang, Y., Liu, R., Zhu, Z. and Zhou, Z., (2018). The putative ß-glucosidase BGL3I regulates cellulase induction in Trichoderma reesei. *Biotechnology for biofuels*, 11(1), 1-14; doi.org/10.1186/s13068-018-1314-6