ORIGINAL PAPER

Co-culturing of Novel *Bacillus* Species Isolated from Municipal Sludge and Gut of Red Wiggler Worm for Improving CMCase Activity

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Abstract

The novel bacterial strains EF2 and OW1-1 were isolated from intestine of *Eisenia fetida* and municipal organic waste respectively. The qualitative screening of strains in carboxymethyl cellulose (CMC) agar plate showed a larger zone of clearance with Gram's iodine staining. The 16S rRNA gene sequences of the strains EF2 and OW1-1 were identified as a gram-positive *Bacillus* sp. The strains exhibited significantly high CMCase activities of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml, respectively, in EF2 and OW1-1 when 2.5% (w/v) of lactose was used as a carbon source at their optimal pH and temperature. The SDS-PAGE and zymogram analysis of crude enzyme revealed that the molecular weight of CMCase was 60 kDa in both strains. In comparing to monoculture of EF2 and OW1-1, the co-culture increased their activity by 15% and 35.71% respectively. The higher CMCase activity of strains in a wider range of temperatures and pH fluctuation could be a good choice for biofuel industries.

Statement of Novelty

This manuscript deals on isolation and characterization of cellulolytic bacteria using 16S rRNA sequences. The cellulolytic bacteria were isolated from various sources and their qualitative screening for cellulase were preformed. The most efficient and closely related *Bacillus* sp. strains EF2 and OW1-1 were further used for quantification of CMCase activity. The comparative CMCase activity of both strains were well documented in regard to optimize their different physicochemical parameters and analyzed the protein bands in SDS-PAGE and zymogram, which is novel to be seen. The results on co-culture of two *Bacillus* sp. in contrast to their monoculture, showed significantly increased in CMCase activity which could be a better choice in the biofuels industries.

Introduction

The biofuels such as bioethanol, biodiesel, biogas, etc. from lignocellulosic biomass are gaining popularity because it is considered as a sustainable, cost efficient, ecofriendly and showed a promising alternative to fossil fuels. The biofuels are renewable energy, has low emission of greenhouse gases and can mitigate the environmental challenges [1, 2], thus able to make a global priority. However, the main hurdles lie in the recalcitrance of its bonding due to protective covering of lignin and crystalline nature of cellulose sheathed by hemicellulose, allowing very less surface area for enzymatic hydrolysis [3–6]. Moreover, the competing land uses versus demand and supply of non-food biomass [7], requirement of multi-disciplinary teams of skilled personals, industrial infancy [8, 9], financial investment and fuels market, etc. in many circumstances are some other bottle neck in higher yield of efficient biofuels. Although, there are several physical, and chemical methods available for disruption of lignocellulosic layers, the biological method has its financial and environmental benefits [4].

Since, cellulose is the most abundant biopolymer found in earth, it has been highly studied in production of biofuels and bioproducts. The bioconversion of cellulosic biomass using cellulase secreted from various bacteria and

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fungi has its greater industrial significance. There are three major enzymatic components of cellulase: endoglucanase, exoglucanases and β-glycosidase; belonging to the glycosyl hydrolase (GH) family. The synergistic activity of these enzymes disintegrates the glycosidic bonds of cellulose. The endo- and exoglucanases act on the chain ends of cellulose to release cellobiose and some sugar molecules whereas the β-glucosidases is essential to break cellobiose into sugars [10, 11]. The β-glucosidase has major role in minimizing the inhibitory effect of cellodextrin and cellobiose by cleaving the final glycosidic bonds of cellobiose releasing sufficient monomeric sugar molecules which in alcoholic fermentation, produce biofuels and value-added products.

This synergism of enzymes can easily observe in the natural environment where the whole microbial consortium secret various lignocellulose degrading enzymes that work synergistically in degrading plant biomass [12] and playing a vital role in regulating the carbon cycle [13]. Thus, it is obvious that mixed populations of microbes with individually optimized populations has ability to break the recalcitrant biopolymers by synergistic action of multiple enzymes which could otherwise become difficult for individual strains [14]. Many efforts have been made in genetic engineering [15–18] to produce robust microorganisms that can survive in extreme condition and continue their higher yield of cellulolytic enzymatic components. However, it is not always possible to produce significant yield of enzymes from single strain of any bacterium. Thus, the culture of two or more species of bacteria in many circumstances is beneficial for efficient enzymatic hydrolysis because they relies in metabolic cross-feeding [19, 20] and produce various cellulase which synergistically work together in cellulose hydrolysis [21]. The strains in co-culture can adapt to the minor fluctuation in culture conditions and degrade the substrate within their intricate network where the whole consortia work together to get the improved enzyme levels in comparison to their pure strains.

The cellulose degrading microbes are found in various places including organic matter decaying sites, on the soil and in the gut of some animals such as insects, earthworm, gastropods, ruminant etc. Several studies have been conducted in isolation of efficient cellulolytic bacterial strains from different natural resources. However, the industrially important, high cellulase yielding strains vigorously active at higher temperature and pH fluctuation are still in demand. Nevertheless, the conventional technique of microbial isolation and screening for new cellulose degrading strain still has its greater significance due to likeliness of getting good strain with additional gene of interest. Thus, it is very essential and important to isolate the high cellulose degrading bacteria for their possible industrial application. This study mainly focused on isolation of cellulolytic bacteria from various sources, optimize the enzyme production from

efficient bacteria, co-culture them for higher yield of cellulolytic components and compare their enzyme activity in an optimum condition.

Materials and Methods

Media Preparation

The solid agar and broth media were prepared for bacterial growth. The composition of culture media was as follow.

Luria–Bertani (LB) medium: 1.0 g peptone, 0.5 g yeast extract, 0.5 g NaCl and distill water up to 100 ml.

LB agar: 1.0 g peptone, 0.5 g yeast extract, 0.5 g NaCl, 1.5 g agar and distill water up to 100 ml.

Carboxymethyl cellulose (CMC) agar: 0.5 g CMC, 0.1 g NaNO$_3$, 0.1 g K$_2$HPO$_4$, 0.1 g KCl, 0.05 g MgSO$_4$, 0.05 g yeast extract, 1.15 g agar and distill water up to 100 ml.

Minimal salt medium: 1% (w/v) CMC and 0.5% (w/v) yeast extract, 0.1 g NaNO$_3$, 0.1 g K$_2$HPO$_4$, 0.1 g KCl, 0.05 g MgSO$_4$ and distill water up to 1000 ml.

Bacterial Isolation and Characterization

Bacterial isolation was done from samples such as soil, rotten wood, paper mill sludge, organic waste, wastewater and gut of earthworm (*Eisenia fetida*) collected from Thunder Bay, ON, Canada. After 10× serial dilution, 200 µl of each sample was inoculated in LB agar plate and incubated at 37 °C for 24 h. The bacterial colonies with morphological and physiological difference developed after 24 h were isolated and transferred into LB medium. The pure bacterial colonies were selected after repetitive streaking followed by isolation and re-culture in LB medium at 37 °C for 24 h.

The cellulolytic efficiency of bacterial strains was confirmed with the qualitative screening for relative carboxymethyl cellulase (CMCase) activity which was followed by DNA extraction. The bacterial DNA of some efficient bacterial strains was extracted using bacterial genomic DNA isolation kit (Norgen Biotek Corp. ON, Canada). The universal forward primer HAD-1 (5′-GACTCCTACGGGAGGCAG CAGT) and reverse primer E1115R (5′-AGGGTTGCGCTC GTTGCGGG) were used in the reaction. The 16S rRNA was amplified using polymerase chain reaction (PCR) followed the manufacturer's instructions (FroggaBio protocol). Briefly, the PCR thermal cycle was adjusted as follows: initial denaturation at 94 °C for 3 min, 30 successive amplification cycles (denaturation: 94 °C for 30 s, annealing: 56 °C for 30 s and extension: 72 °C for 1 min) and final extension at 72 °C for 10 min. The DNA was purified using Gel/PCR DNA fragments extraction kit (Geneaid, FroggaBio). The purified 16S rRNA samples were sent to sequencing lab. The sequence results were BLAST using National Center

for Biotechnology Information (NCBI) database. The phylogeny was evaluated by multiple alignment of sequences in phylogeny.fr, a web-based tree view software [22].

Preparation of Seed Culture and Size of Inoculum

The seed culture for quantitative assay was prepared from stock culture in agar plate by transferring strains into the tube containing 5 ml LB medium using inoculation loop. The LB medium seed culture was incubated in shaking incubator at 37 °C and 200 rpm for 24 h. The proportion of inoculum size was maintained at 1:50 ratio where 1 ml LB seed culture was transferred into a 250 ml Erlenmeyer flask containing 50 ml minimal salt medium in each batch fermentation.

Qualitative Cellulase Assay

The overnight cultured bacterial strains in LB medium (5 μl) was inoculated at the centre of CMC agar plate (containing 0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract, 1.15 g agar and distill water up to 100 ml). The agar plate was kept in incubator at 37 °C for 48 h. Qualitative screening of cellulolytic bacterial strain was performed with Gram's iodine test [23]. The CMC plate was filled with 3% (w/v) Gram's iodine solution and observed the zone of clearance after 2 min. The cellulase produced from bacteria degrade the cellulosic content of agar plate into some monosaccharides and disaccharides which give zone of clearance (halo) with the iodine solution

[24]. The halo measurements were compared with *E. coli* and cellulase (dilution—2 mg/ml) from *T. reesei* ATCC 26921 (Sigma Aldrich—C2730, Canada) for negative and positive controls respectively. The experiments were conducted in triplicates and the halo diameters in mm were recorded. The strains with higher enzymatic index (EI) values (Fig. 1) were further analyzed for their characterization and quantitative CMCase activities.

Quantitative Cellulase Assay

One milliliter of overnight cultured LB medium seed culture was transferred into a 250 ml flask. Each flask containing 50 ml minimal salt medium with 1% CMC as a substrate was used for bacterial growth and enzyme production, keeping a constant agitation of 200 rpm throughout the experiment. The physiochemical parameters (including temperature, pH, carbon and nitrogen sources) were optimized by considering one parameter at a time. Since the experiments were conducted in Erlenmeyer flask, the initial pH measurement of culture medium has been done before the inoculation of strains. The effect of initial pH, temperature and incubation time on CMCase production was measured by culturing the strains in different pH ranges from 5 to 9 at temperature of 30–50 °C for 4 days. Similarly, the effect of different carbon sources (including CMC, D-glucose, D-fructose, D-sorbitol and D-lactose) and nitrogen sources (including peptone, urea, yeast extract and $(NH_4)_2SO_4$) on enzyme production were also evaluated. The culture broth of 1 ml was harvested each 24 h interval of time and centrifuged at 12,000×*g* for

Enzymatic index:

KF11: 3.26
OW1-1: 3.46
P4: 2.97
P5-2: 3.16
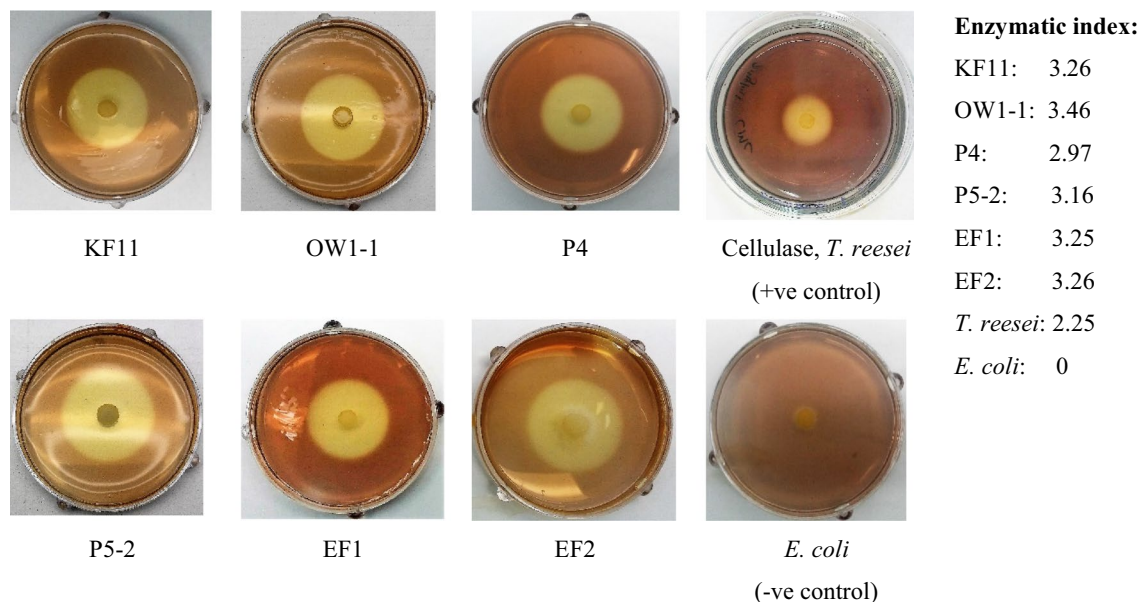EF1: 3.25
EF2: 3.26
T. reesei: 2.25
E. coli: 0

Fig. 1 Plates having zone of clearance (halo) in Gram's iodine test for six cellulase producing isolates and their EI values were compared with *E. coli* as a negative control and cellulase from *T. reesei* ATCC 26921 as a positive control

3 min. The supernatant was collected for extracellular crude enzyme and analyzed the enzyme activity with the measurement of reducing sugars yield by 3,5-dinitro-salicylic acid (DNS) method [25] with some modification. Briefly, the reaction mixture containing aliquot (50 μl) of crude enzyme from supernatant and 50 μl of 0.5 M citrate buffer (pH 6.0) with 0.5% CMC were transferred into a 1 ml microcentrifuge tube. The reaction mixture was incubated in hot water bath at 55 °C for 30 min. The DNS solution of 200 μl was added to the reaction mixture and the tube was kept in boiling water bath for 5 min. The reducing sugars liberated in reaction mixture were estimated by DNS method using glucose standard curve. The absorbance was measured in room temperature at OD 540 nm in a micro-plate reader spectrophotometer (BioTek, USA). The bacterial growth was measured in term of biomass (absorbance at OD 600) whereas the enzyme activity was expressed in IU/ml, where one unit of CMCase enzyme corresponds to the release of 1 μM of reducing sugar equivalent per minute from substrate.

Effect of Co-culture on Enzyme Production

The seed culture of both bacterial strains, EF2 and OW1-1 were prepared separately in LB medium. A total volume of 1 ml seed culture was maintained by transferring 500 μl of each overnight cultured strain into a 250 ml flask containing minimal salt medium with 1% (w/v) CMC. The enzyme activity was estimated in optimum condition by harvesting 1 ml of sample from the culture medium following similar procedures of enzyme activity assay.

SDS-Polyacrylamide Gel Electrophoresis

The cellulase enzyme was separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The hydrolytic activity of cellulase was observed in zymogram. The crude enzyme samples from strain EF2 and OW1-1 were run along with protein ladder (Bio Basic, Canada) in 10% of acrylamide gel. A constant supply of 120 V was maintained until the sample crossed the stacking gel, while the 160 V was maintained in the separating gel. The gel was cut into two parts, one of which was used to detect the protein while the other half used to perform the zymogram. One-half of gel was immersed in Coomassie Brilliant Blue for 45 min and de-stained with decolouring buffer until the bands were prominent. Whereas the other half of gel was used to observe the hydrolytic activity after washed with 1% (v/v) Triton X-100. The gel was immersed in 0.5 M citrate buffer (pH 6.0) with 0.5% CMC and incubated in hot water bath (55 °C) for 30 min followed by stained in 0.1% Congo red for 30 min. 1 M sodium chloride solution was used to distain the gel and was treated with 4% (w/v) acetic acid solution to make a prominent hydrolytic band

in zymogram analysis. The presence of protein bands and hydrolysis bands observed in the gel was compared with standard protein marker (Bio Basic, Canada) to estimate the molecular weight of cellulase.

Statistical Analysis

All the experimental data were obtained in the form of triplicates and results were expressed in terms of mean ± standard deviation (SD). The mean values of triplicates were analyzed by one way analysis of variance (ANOVA) followed by two tailed t test using corresponding confidence level of 95% (i.e. P value at < 0.05). The multiple comparison among the different variables were made by the post-hoc Bonferroni correction of t test values at less than P/n level of significance.

Results and Discussion

Qualitative CMCase Activity Assay

Out of 26 isolates tested in Gram's iodine, 25 strains showed their positive results in plate assay with various sizes of halo. Only six of the strains with larger halos (Fig. 1) in CMC plates were selected for further characterization. The halo sizes were recorded (diameter shown in Fig. 2) and compared with *E. coli* and cellulase from *T. reesei* ATCC 26921 for negative and positive controls respectively. The strains EF2 isolated from intestine of red wiggler worm (*E. fetida*) and OW1-1 isolated from municipal organic waste sludge showed larger halo sizes and EI. Moreover, the strains EF2 and OW1-1 also showed a proximity in their phylogenic relationship (Fig. 2) thus, were selected for their further quantification of CMCase activity.

DNA Extraction and Characterization of Bacterial Strains

The strains with larger halos observed in qualitative plate assay were selected for DNA extraction. The bacterial DNA of six strains were isolated using bacterial genomic DNA isolation kit (Norgen Biotek Corp) and amplified its 16S rRNA using PCR (FroggaBio) following their protocols. The amplified DNA fragments were run in 1% agarose gel for validation of 16S rRNA which showed the clear bands of about 800 bp. It was purified using Geneaid PCR/Gel purification kit and sent to Eurofins Genomics for sequencing. The nucleotide sequences of strains were successfully uploaded in NCBI database and the accession numbers were obtained. The strains were identified as gram +ve bacteria of genus *Bacillus* and *Lysinibacillus* with 97–99% homology. Both the strains EF2 and OW1-1 were identified as *Bacillus* sp. The GenBank accession numbers of *Bacillus*
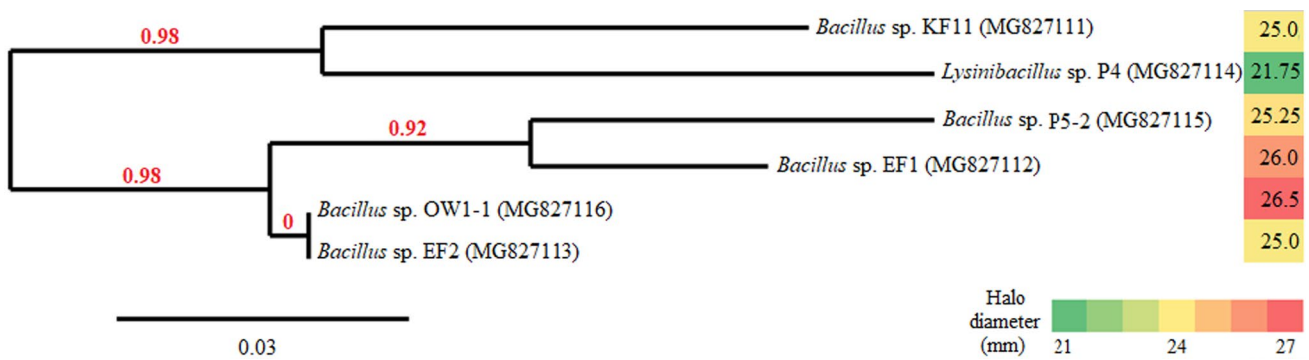
Fig. 2 Phylogeny of bacterial strains made from multiple alignments of sequences in phylogeny.fr software. The red coloured number represents a branch support values, the accession numbers are given in the brackets. Colour scale on the right shows the size of halo (mm) in CMC agar plates. (Color figure online)

sp. strains EF2 and OW1-1 were obtained as MG827113 and MG827116 respectively. The web-based phylogeny.fr software was used for making tree view (Fig. 2) of phylogeny which showed the strains EF2 and OW1-1 have proximity in phylogeny, and thus could be suitable for co-culture.

Effect of Incubation Time on Enzyme Production

The strains EF2 and OW1-1 were separately cultured in 250 ml conical flask containing 50 ml minimal salt medium for 4 days. The bacterial culture of 1 ml was harvested every 12 h for 4 days to analyze the biomass and enzyme activity. The bacterial growth was measured in terms of biomass at OD 600 nm and the reducing sugars formed was estimated in OD 540 nm. The higher CMCase production was observed in 24 h which decreased with increases in incubation time (Fig. 3). The activity from 24 h of incubation was significantly different than other incubation times with the $P < 0.05$ for both strains. It was in harmony with the earlier finding where rapid increase in production of endoglucanase was recorded from 6 h, which reached a maximum at 24 h and steadily decrease thereafter [26].

Effect of Temperature and pH on Enzyme Production

The enzyme production was greatly influenced by temperature and pH. Different bacteria have their own optimum temperature requirement for maximum enzyme production. The strains were cultured in 50 ml of minimal salt medium at 30, 35, 40, 45 and 50 °C temperature. These strains are mesophilic bacteria which preferred moderate temperature of 40 °C for maximum CMCase production. The mesophilic *Bacillus subtilis* and *Bacillus circulans* gave maximum yield of cellulase at 40 °C [27]. Some other bacteria such as *Bacillus, Cellulomonas* and *Micrococcus* sp. showed their higher endoglucanase activity in 40 °C [28]. The relative CMCase activities of EF2 and OW1-1 were calculated in comparison to their maximum observed activity. The CMCase production was high in 40 °C and its activity was significantly different than other temperatures with the $P < 0.05$ for both strains (Fig. 4). The activity is decreased with change in
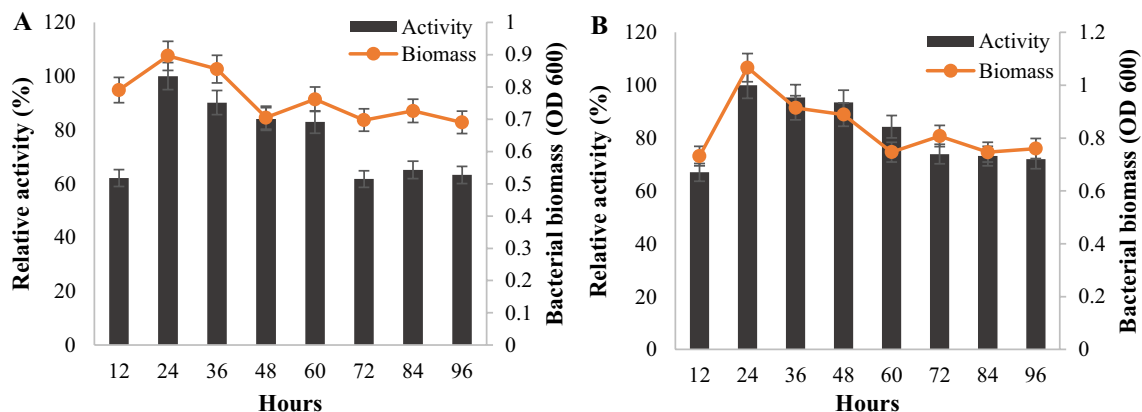

Fig. 3 Effect of incubation time in biomass and CMCase activity by strain EF2 (**a**) and OW1-1 (**b**)
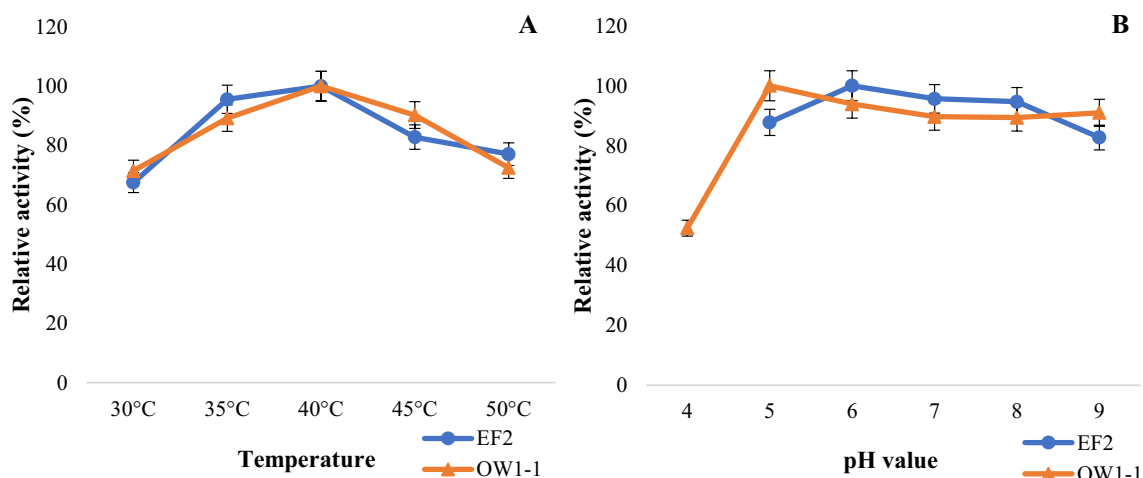
Fig. 4 Effect of temperature (**a**) and pH (**b**) in CMCase activity by strains EF2 and OW1-1

temperature perhaps due to inhibition of cellulase multienzyme complex system.

The effect of pH on CMCase production was measured in optimum 40 °C temperature and 24 h incubation time. The pH was adjusted to 4, 5, 6, 7, 8 and 9 using 2 N KOH and 0.2 N HCl. The bacterial strains EF2 and OW1-1 have better CMCase production in the broader pH ranges from 5 to 7 however the highest activity observed in pH 6 and pH 5 respectively. Similar optimum pH 5.7–6.1 was reported in *Clostridium thermocellum* [29]. The activity gradually decreased with further change in pH values. The optimum activity was significantly different than other pH values with the $P < 0.05$ for both strains. The bacteria such as *Micrococcus* sp. SAMRC-UFH3 [30], *Clostridium straminisolvens* strain CSK1 [31] decreased their activity with changes in optimum pH. However, some of the industrially important *Bacillus* sp. give better enzyme activity at wider pH ranges [32–34]. For these bacteria, minor fluctuation in acidic environment does not necessarily affect their cellulolytic capacity.

Effect of Nitrogen on Enzyme Production

The CMCase production has been greatly affected by various sources of nitrogen. We tested the effect of 0.5% w/v of each $(NH_4)_2SO_4$, peptone, urea, yeast extract in enzyme production at their respective optimum pH (as pH 6 for EF2 and pH 5 for OW1-1), temperature of 40 °C and 24 h incubation time. The result showed significantly higher yield of CMCase when yeast extract was used as a source of nitrogen. It was followed by peptone, $(NH_4)_2SO_4$ and the least activity observed in urea in both strains (Figs. 5, 6). The optimum CMCase activity was significantly different with the $P < 0.05$ for both strains. The yeast extract and peptone are organic nitrogen sources which can results in better cellulase production [35, 36]. However, the lower activity
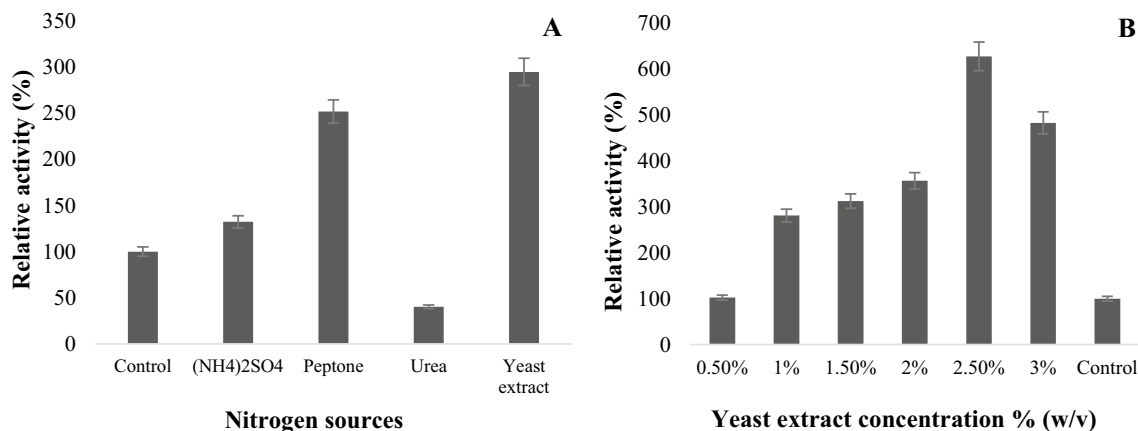

Fig. 5 Effect of nitrogen sources (**a**) and yeast extract concentration (**b**) on CMCase activity by strain EF2
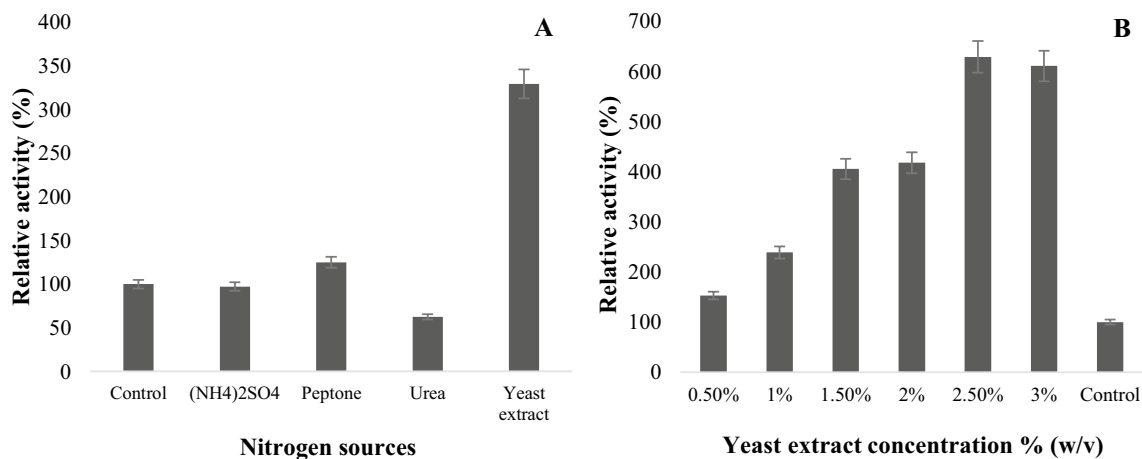
Fig. 6 Effect of nitrogen sources (**a**) and yeast extract concentration (**b**) on CMCase activity by strain OW1-1

observed in inorganic nitrogenous sources (such as urea and ammonium sulphate) might be due to the inhibitory effect of medium acidification resulted from metabolism of inorganic nitrogen which in turn affects cellulase production [36].

The gradual increase in yeast extract concentration in minimal salt medium increases the enzyme production until it reaches to the optimum 2.5% (w/v) in both strains. The results of ANOVA (with the $P > 0.05$) showed that the optimum activity was not significantly different than other concentrations of yeast extract for both strains. Since, nitrogen is one of the major constituents of proteins, the bacteria are sensitive to the types and concentration of nitrogen sources which are specific to the species. Thus, the bacterial growth and enzyme production for dissimilar species differ dramatically with respect to their optimum condition. However, similar high CMCase activity was observed in *Bacillus* sp. C1AC55.07 when 2% yeast extract was used [37]. The highest CMCase activity in yeast extract among both strains EF2 and OW1-1 may be due to the presence of some other supplemental elements such as vitamin, trace nutrients, sulfur, etc. in yeast extract which are suitable for bacterial growth [38].

Effect of Carbon on Enzyme Production

The strains EF2 and OW1-1 can utilize various carbon sources for CMCase production. Both strains were grown separately in minimal salt medium containing 1% (w/v) of each CMC, glucose, fructose, lactose and sorbitol in their respective optimum culture condition. The presence of lactose in culture medium gave a significantly higher CMCase production then other carbon sources. This CMCase production was gradually increased with increase in lactose concentration and attained its maximal production at 2.5% (w/v) concentration in the medium. The CMCase

activity of 13.742 ± 0.09 IU/ml and 12.812 ± 0.07 IU/ml were recorded in EF2 and OW1-1 respectively. However, the highest CMCase activity of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml were recorded from EF2 and OW1-1 respectively when 2.5% (w/v) lactose was used with their optimum culture condition (Figs. 7, 8). The results of ANOVA with the *P*-values of 0.0019 and 0.0016 respectively for EF2 and OW1-1 (i.e. $P < 0.05$) showed that the optimum enzyme activity was significantly different than other concentrations of lactose in both strains. Similar higher CMCase activity in lactose as a carbon source was observed in *Microbacterium* sp. [39], *Bacillus* sp. strain K1 [40], *Aspergillus hortai* [41]. It might be due to the lactose-induced mechanism of cellulase production [42] which could be helpful to improve its industrial application [39].

Effect of Co-culture on Enzyme Production

The monoculture of strains EF2 and OW1-1 have shown their higher CMCase production at 40 °C but at dissimilar optimum pH 6 and pH 5 respectively. Thus, the initial pH for co-culture was optimized by co-culturing the strains in 50 ml minimal salt medium containing 1% CMC with pH 5, 6, 7, 8 and 9 at an optimum 40 °C temperature. The higher CMCase activity of 1.925 ± 0.005 IU/ml was recorded when co-culture was provided with 1% CMC as a carbon source at pH 6. The activity was significantly different ($P < 0.05$) than other pH values (Fig. 9). It was a minimal increased in value in comparison to their pure monoculture. However, the CMCase activities of co-culture (i.e. 40.605 ± 0.04 IU/ml) was 15% and 35.71% higher than monoculture of pure strains EF2 (i.e. 35.307 ± 0.08 IU/ml) and OW1-1 (i.e. 29.92 ± 0.01 IU/ml) respectively in optimum lactose concentration (Fig. 10). Similarly, the CMCase activity in co-culture was significantly higher than pure strain OW1-1
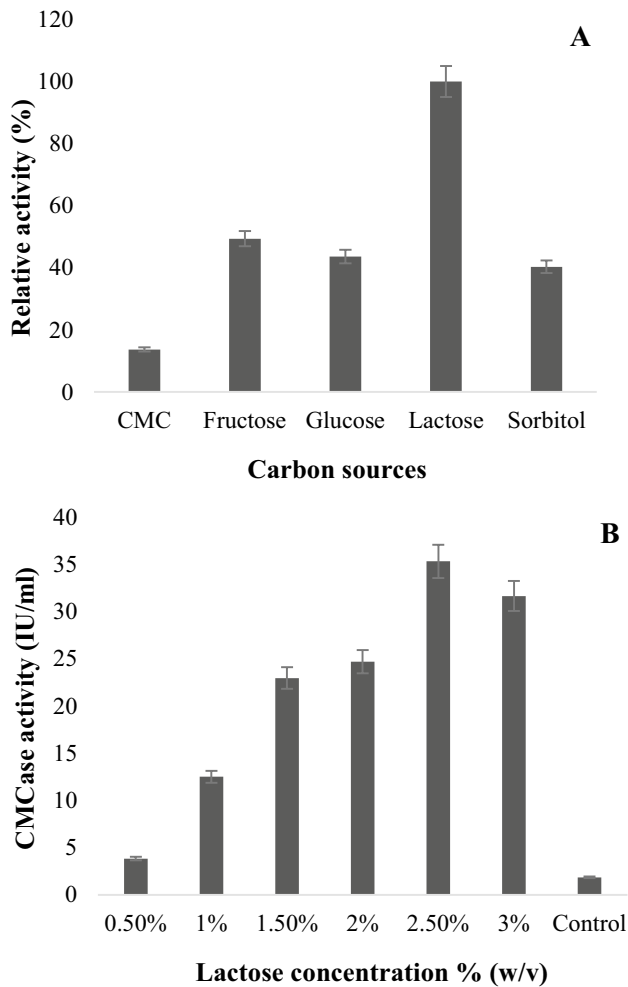
Fig. 7 Effect of carbon sources (**a**) and lactose concentration (**b**) on CMCase activity by strain EF2

when optimum yeast extract concentration was used. Generally, the microbial consortia are better adapted to minor fluctuation in pH and temperature [43]. Several white rot fungi (such as *Fusarium* sp., *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Pleurotus ostreatus*, etc.) and some bacterial strains of the genus *Clostridium* are commonly practiced in co-culture [43]. An efficient lignocellulolytic enzymes complex has been reported by constructing composite microbial system from mesophilic bacteria belonging to the genera *Clostridium, Bacteroides, Alcaligenes, Pseudomonas*, etc. [44]. The consortium showed the high performance in degradation of lignocellulosic biomass due to synergistic enzymes at optimum temperature and pH of 40 °C and 6.0 respectively [44]. The engineered microbial consortia are usually adapted to environmental fluctuation and known to perform complex functions that are difficult to individual populations [14]. However, there are several other limiting factors such as variation in substrate utilization, nutritional requirement, dissimilar requirement of ionic concentration in culture medium, different genetic makeup, varied enzymatic components, diverse ecological niche and adaptational factors etc. could play a vital role in metabolic functions of microorganisms thereby affected in enzyme production. Thus, the maximum increase in yield of enzymatic components can be achieved after careful consideration of as much factors as possible.

SDS-PAGE and Zymogram

The protein bands of CMCase was observed in 10% acrylamide gel. The hydrolytic band of CMCase clearly indicated the cellulolytic activity in zymogram. It was run under the same conditions of SDP-PAGE which correspond to ~ 60 kDa in both strains EF2 and OW1-1 (Fig. 11). A
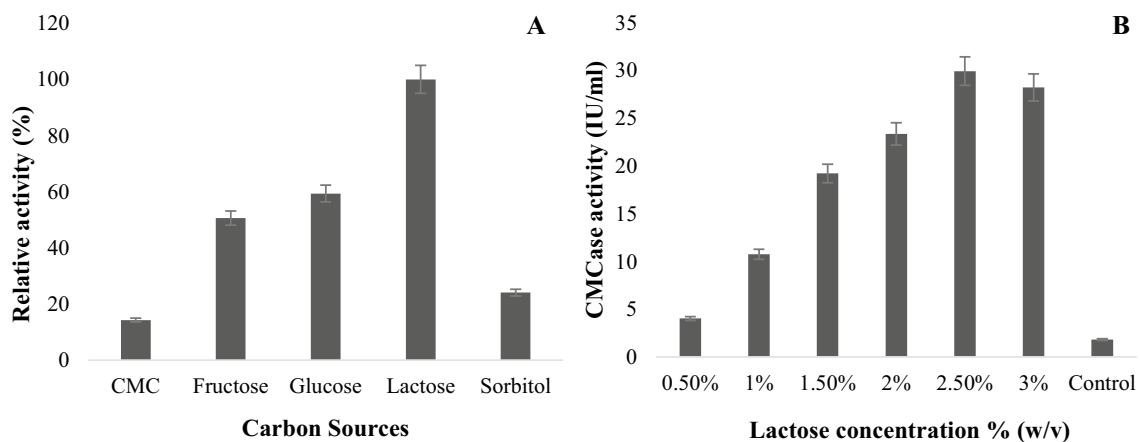

Fig. 8 Effect of carbon sources (**a**) and lactose concentration (**b**) on CMCase activity by strain OW1-1
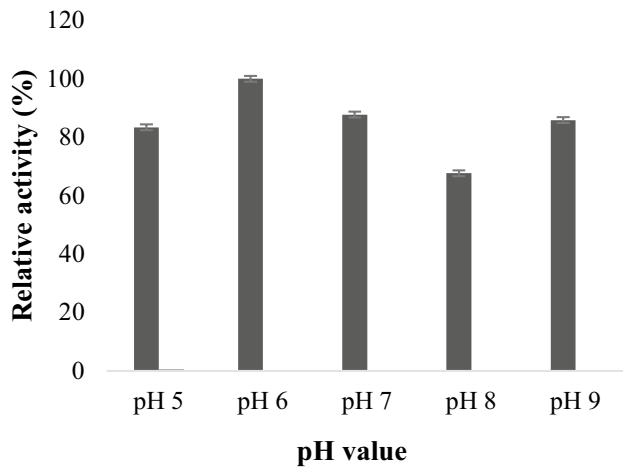
Fig. 9 Effect of pH in CMCase activity by co-culture of strain EF2 and OW1-1

different among the variables with their optimum enzyme activity in *Bacillus* sp. strain EF2 and OW1-1 respectively (Fig. 12). The post-hoc Bonferroni correction of two tail *t* test comparison demonstrated that the pH 6 has significantly higher (i.e. $P/n < 0.01$) activity than incubation time (*) that showed the strain EF2 preferred an acidic medium for optimum enzyme production. Similar, significantly high (i.e. $P/n < 0.01$) enzyme activity in lactose concentration than all other variables (**) showed that the optimum lactose concentration contribute as a major carbon source induced for maximum CMCase production in both *Bacillus* sp. strain EF2 and OW1-1.
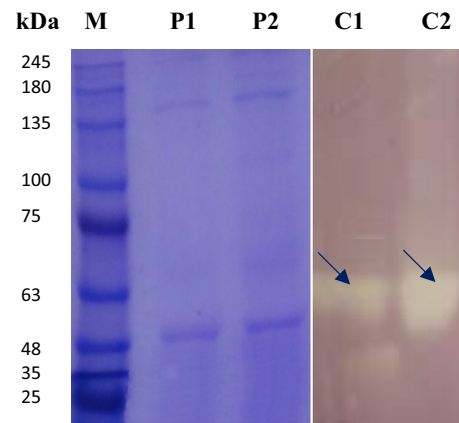
relatively a higher molecular weight of 80 kDa has been reported in *Bacillus vallismortis* RG-07 [34], and 83 kDa and 50 kDa CMCase in wild type strain of *Aspergillus niger* [45]. However, the CMCase with molecular weight of 55 kDa was observed in *Bacillus* sp. C1AC5507 [46]. Similarly, the cellulase bands in the range of 30–65 kDa in *Bacillus pumilus* EB3 [47] and 60 kDa in *A. niger* [48] have been estimated from SDS-PAGE.

Statistical Analysis

The comparison of enzyme activity among different independent variables (including incubation time, temperature, pH, lactose concentration and yeast extract concentration) were first checked for its significance with ANOVA followed by post-hoc Bonferroni correction of *t* test values. The ANOVA result with the *P* value of 0.000012 and 0.000002 (i.e. $P < 0.05$) showed that there is significant

Fig. 11 SDS-PAGE and zymogram of crude CMCase from strain EF2 and OW1-1. (M protein marker, P1 and P2 protein bands of EF2 and OW1-1 respectively, C1 and C2 CMCase hydrolytic bands of EF2 and OW1-1 respectively in zymogram). The molecular weight of CMCase was estimated about 60 kDa in both strains
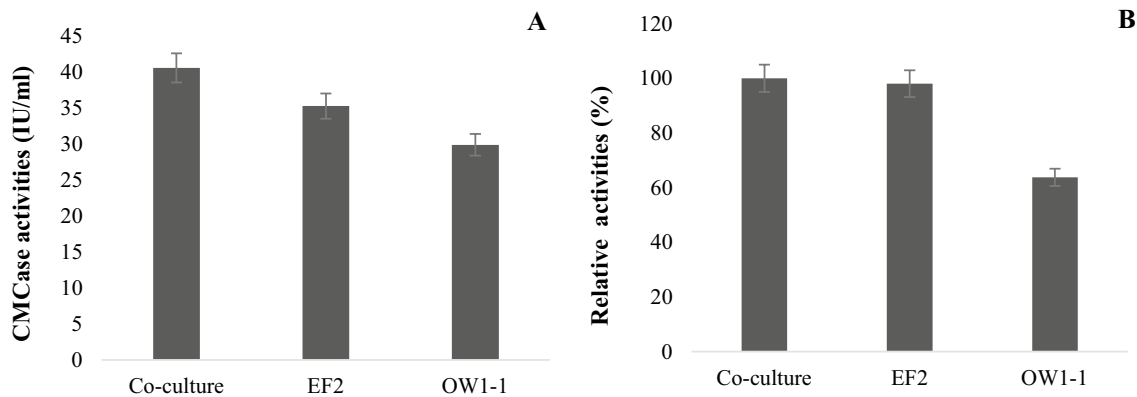

Fig. 10 CMCase activities of mono and co-culture in optimum concentration of 2.5% (w/v) of lactose (**a**) and 2.5% (w/v) of yeast extract (**b**)
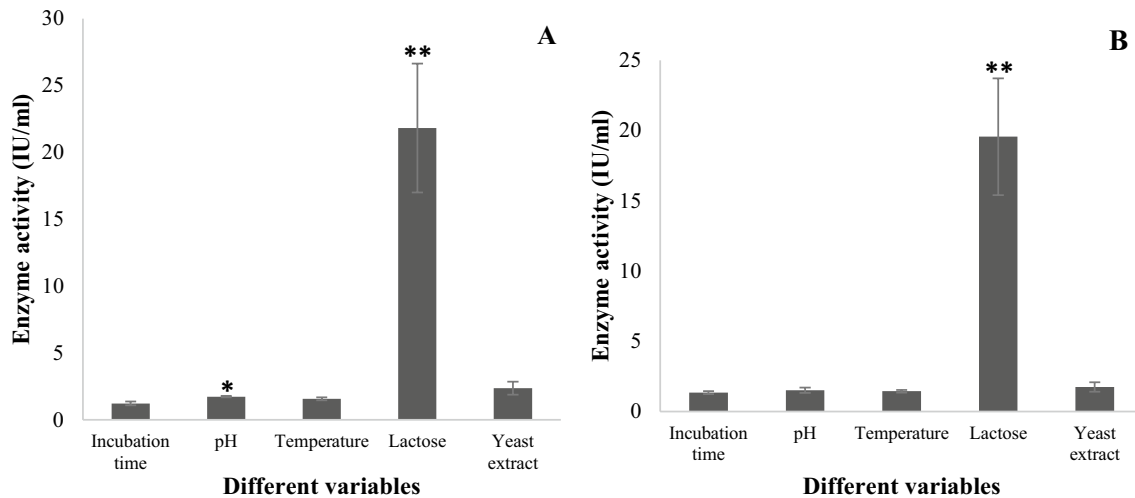
Fig. 12 ANOVA was significant ($P < 0.05$) in multiple comparison of independent variables and the post-hoc Bonferroni correction of t test showed significantly different between pH and incubation time (*) in *Bacillus* sp. strain EF2, and lactose concentration and all other variables (**) at $P < 0.01$ (i.e. *P*/n) level of significance in *Bacillus* sp. strain EF2 (**a**) and strain OW1-1 (**b**)

Conclusion

Some of the efficient cellulolytic bacteria can degrade the crystalline cellulose and release monomeric sugar molecules. These sugars can be converted to biofuels and value-added products after alcoholic fermentation. Thus, the isolation of such efficient bacteria is a fundamental key step of biofuel industries. Six cellulolytic bacteria were isolated from different sources including soil, rotten wood, organic waste, paper mill sludge and gut of earthworm. The 16S rRNA sequence identified the strains were of gram +ve bacteria belonging to genus *Bacillus* and *Lysinibacillus*. Two efficient *Bacillus* sp. strains EF2 and OW1-1 were selected for comparative enzyme activity assay in monoculture and co-culture. The presence of yeast extract and lactose in the culture medium induced the higher enzyme activity. The monoculture of strains EF2 and OW1-1 showed significantly increased (P < 0.05 level of significance) CMCase activity of 35.307 ± 0.08 IU/ml and 29.92 ± 0.01 IU/ml with lactose in the culture medium at 40 °C and optimum pH 6 and pH 5 respectively. The co-culture of these *Bacillus* sp. produced 15% and 35.71% higher CMCase activity than monoculture of EF2 and OW1-1 respectively. According to the hydrolytic activity shown in zymogram the molecular weight of CMCase was 60 kDa. The strains showed greater enzyme activity in broad range of temperatures (from 35 to 45 °C) in acidic pH which suggest that the *Bacillus sp.* strains EF2 and OW1-1 could be the potential cellulolytic candidates for biofuel industry.

References

1. Medipally, S.R., Yusoff, F.M., Banerjee, S., Shariff, M.: Microalgae as sustainable renewable energy feedstock for biofuel production. Biomed Res. Int. **2015**, 1–13 (2015). https://doi.org/10.1155/2015/519513

2. Singh Nigam, P., Singh, A.: Production of liquid biofuels from renewable resources. Prog. Energy Combust. Sci. **37**, 52–68 (2011). https://doi.org/10.1016/j.pecs.2010.01.003

3. Panwar, N.L., Kaushik, S.C., Kothari, S.: Role of renewable energy sources in environmental protection: a review. Renew. Sustain. Energy Rev. **15**, 1513–1524 (2011). https://doi.org/10.1016/j.rser.2010.11.037

4. Sharma, H.K., Xu, C., Qin, W.: Biological pretreatment of lignocellulosic biomass for biofuels and bioproducts: an overview. Waste Biomass Valoriz. (2017). https://doi.org/10.1007/s12649-017-0059-y

5. Horn, S., Vaaje-Kolstad, G., Westereng, B., Eijsink, V.G.: Novel enzymes for the degradation of cellulose. Biotechnol. Biofuels **5**, 45 (2012). https://doi.org/10.1186/1754-6834-5-45

6. Zhang, Y.-H.P., Ding, S.-Y., Mielenz, J.R., Cui, J.-B., Elander, R.T., Laser, M., Himmel, M.E., McMillan, J.R., Lynd, L.R.: Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnol. Bioeng. **97**, 214–223 (2007). https://doi.org/10.1002/bit.21386

7. Harvey, M., Pilgrim, S.: The new competition for land: food, energy, and climate change. Food Policy **36**, S40–S51 (2011). https://doi.org/10.1016/j.foodpol.2010.11.009

8. FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A.: A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour. Technol. **101**, 8915–8922 (2010). https://doi.org/10.1016/j.biortech.2010.06.125

9. Zhu, L.: Biorefinery as a promising approach to promote microalgae industry: an innovative framework. Renew. Sustain. Energy Rev. **41**, 1376–1384 (2015). https://doi.org/10.1016/j.rser.2014.09.040

10. Maki, M., Leung, K.T., Qin, W.: The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass.

Int. J. Biol. Sci. **5**, 500–516 (2009). https://doi.org/10.7150/ijbs.5.500

11. Dashtban, M., Maki, M., Leung, K.T., Mao, C., Qin, W.: Cellulase activities in biomass conversion: measurement methods and comparison. Crit. Rev. Biotechnol. **30**, 302–309 (2010). https://doi.org/10.3109/07388551.2010.490938

12. Hatakka, A., Hammel, K.E.: Fungal biodegradation of lignocelluloses. In: Hofrichter, M. (ed.) Industrial Applications, pp. 319–340. Springer, Berlin (2011)

13. Lindahl, B.O., Taylor, A.F.S., Finlay, R.D.: Defining nutritional constraints on carbon cycling in boreal forests – towards a less `phytocentric' perspective. Plant Soil **242**, 123–135 (2002). https://doi.org/10.1023/A:1019650226585

14. Brenner, K., You, L., Arnold, F.H.: Engineering microbial consortia: a new frontier in synthetic biology. Trends Biotechnol. **26**, 483–489 (2008). https://doi.org/10.1016/j.tibtech.2008.05.004

15. Druzhinina, I.S., Kubicek, C.P.: Genetic engineering of *Trichoderma reesei* cellulases and their production. Microb. Biotechnol. **10**, 1485–1499 (2017). https://doi.org/10.1111/1751-7915.12726

16. Chambergo, F.S., Bonaccorsi, E.D., Ferreira, A.J.S., Ramos, A.S.P., Ferreira Júnior, J.R., Abrahão-Neto, J., Farah, J.P.S., El-Dorry, H.: Elucidation of the metabolic fate of glucose in the filamentous fungus *Trichoderma reesei* using expressed sequence tag (EST) analysis and cDNA microarrays. J. Biol. Chem. **277**, 13983–13988 (2002). https://doi.org/10.1074/jbc.M107651200

17. Chen, J., Zhang, W., Tan, L., Wang, Y., He, G.: Optimization of metabolic pathways for bioconversion of lignocellulose to ethanol through genetic engineering. Biotechnol. Adv. **27**, 593–598 (2009). https://doi.org/10.1016/j.biotechadv.2009.04.021

18. Ellilä, S., Fonseca, L., Uchima, C., Cota, J., Goldman, G.H., Saloheimo, M., Sacon, V., Siika-aho, M.: Development of a low-cost cellulase production process using *Trichoderma reesei* for Brazilian biorefineries. Biotechnol. Biofuels **10**, 30 (2017). https://doi.org/10.1186/s13068-017-0717-0

19. Pande, S., Shitut, S., Freund, L., Westermann, M., Bertels, F., Colesie, C., Bischofs, I.B., Kost, C.: Metabolic cross-feeding via intercellular nanotubes among bacteria. Nat. Commun. **6**, 6238 (2015). https://doi.org/10.1038/ncomms7238

20. Jia, X., Liu, C., Song, H., Ding, M., Du, J., Ma, Q., Yuan, Y.: Design, analysis and application of synthetic microbial consortia. Synth. Syst. Biotechnol. **1**, 109–117 (2016). https://doi.org/10.1016/j.synbio.2016.02.001

21. Zhou, S., Ingram, L.O.: Synergistic hydrolysis of carboxymethyl cellulose and acid-swollen cellulose by two endoglucanases (CelZ and CelY) from *Erwinia chrysanthemi*. J. Bacteriol. **182**, 5676–5682 (2000). https://doi.org/10.1128/JB.182.20.5676-5682.2000

22. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., Gascuel, O.: Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. **36**, W465–W469 (2008). https://doi.org/10.1093/nar/gkn180

23. Kasana, R.C., Salwan, R., Dhar, H., Dutt, S., Gulati, A.: A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr. Microbiol. **57**, 503–507 (2008). https://doi.org/10.1007/s00284-008-9276-8

24. Gohel, H.R., Contractor, C.N., Ghosh, S.K., Braganza, V.J.: A comparative study of various staining techniques for determination of extra cellular cellulase activity on carboxy methyl cellulose (CMC) agar plates. Int. J. Curr. Microbiol. Appl. Sci. **3**, 261–266 (2014)

25. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**, 426–428 (1959). https://doi.org/10.1021/ac60147a030

26. Seo, J.K., Park, T.S., Kwon, I.H., Piao, M.Y., Lee, C.H., Ha, J.K.: Characterization of cellulolytic and xylanolytic enzymes of *Bacillus licheniformis* JK7 isolated from the rumen of a native Korean goat. Asian-Australas. J. Anim. Sci. **26**, 50–58 (2013). https://doi.org/10.5713/ajas.2012.12506

27. Ray, A.K., Bairagi, A., Sarkar Ghosh, K., Sen, S.K.: Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyol. Piscat. **37**, 47–53 (2007)

28. Immanuel, G., Dhanusha, R., Prema, P., Palavesam, A.: Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int. J. Environ. Sci. Technol. **3**, 25–34 (2006). https://doi.org/10.1007/BF03325904

29. Johnson, E.A., Sakajoh, M., Halliwell, G.: Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. **43**, 1125–1132 (1982)

30. Mmango-Kaseke, Z., Okaiyeto, K., Nwodo, U., Mabinya, L., Okoh, A.: Optimization of cellulase and xylanase production by *Micrococcus* species under submerged fermentation. Sustainability **8**, 1168 (2016). https://doi.org/10.3390/su8111168

31. Jungang, W., Binbin, H., Xiaofen, W., Zongjun, C.: Characteristics of cellulase in cellulose-degrading bacterium strain *Clostridium straminisolvens* (CSK1). Afr. J. Microbiol. Res. **11**, 414–421 (2017). https://doi.org/10.5897/AJMR2016.8357

32. Kim, J.-Y., Hur, S.-H., Hong, J.-H.: Purification and characterization of an alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. Biotechnol. Lett. **27**, 313–316 (2005). https://doi.org/10.1007/s10529-005-0685-5

33. Samiullah, T.R., Bakhsh, A., Rao, A.Q., Naz, M., Saleem, M.: Isolation, purification and characterization of extracellular β-glucosidase from *Bacillus* sp. Adv. Environ. Biol. **3**, 269–277 (2009)

34. Gaur, R., Tiwari, S.: Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC Biotechnol. **15**, 19 (2015). https://doi.org/10.1186/s12896-015-0129-9

35. Enari, T., Markkanen, P.: Production of cellulolytic enzymes by fungi. Adv. Biochem. Eng. **5**, 1–24 (1977)

36. Yang, W., Meng, F., Peng, J., Han, P., Fang, F., Ma, L., Cao, B.: Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. Electron. J. Biotechnol. **17**, 262–267 (2014). https://doi.org/10.1016/j.ejbt.2014.08.002

37. Dias, P.V.S., Ramos, K.O., Padilha, I.Q.M., Araújo, D.A.M., Santos, S.F.M., Silva, F.L.H.: Optimization of cellulase production by *Bacillus* sp. isolated from sugarcane cultivated soil. Ital. Assoc. Chem. Eng. **38**, 277–282 (2014). https://doi.org/10.3303/CET1438047

38. Grant, C.L., Pramer, D.: Minor element composition of yeast extract. J. Bacteriol. **84**, 869–870 (1962)

39. Sadhu, S., Saha, P., Mayilraj, S., Maiti, T.K.: Lactose-enhanced cellulase production by *Microbacterium* sp. isolated from fecal matter of zebra (*Equus zebra*). Curr. Microbiol. **62**, 1050–1055 (2011). https://doi.org/10.1007/s00284-010-9816-x

40. Paudel, Y.P., Qin, W.: Characterization of novel cellulose-producing bacteria isolated from rotting wood samples. Appl. Biochem. Biotechnol. **177**, 1186–1198 (2015). https://doi.org/10.1007/s12010-015-1806-9

41. El-Hadi, A.A., El-Nour, S.A., Hammad, A., Kamel, Z., Anwar, M.: Optimization of cultural and nutritional conditions for carboxymethylcellulase production by *Aspergillus hortai*. J. Radiat. Res. Appl. Sci. **7**, 23–28 (2014). https://doi.org/10.1016/j.jrras.2013.11.003

42. Karaffa, L., Fekete, E., Gamauf, C., Szentirmai, A., Kubicek, C.P., Seiboth, B.: D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates. Microbiology **152**, 1507–1514 (2006). https://doi.org/10.1099/mic.0.28719-0

43. Poszytek, K., Ciezkowska, M., Sklodowska, A., Drewniak, L.: Microbial consortium with high cellulolytic activity (MCHCA) for enhanced biogas production. Front. Microbiol. **7**, 324 (2016). https://doi.org/10.3389/fmicb.2016.00324

44. Guo, P., Zhu, W., Wang, H., Lü, Y., Wang, X., Zheng, D., Cui, Z.: Functional characteristics and diversity of a novel lignocelluloses degrading composite microbial system with high xylanase activity. J. Microbiol. Biotechnol. **20**, 254–264 (2010)

45. Coral, G., Arikan, B., Ünaldi, M., Guvenmez, H.: Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. Turk. J. Biol. **26**, 209–213 (2002)

46. Padilha, I.Q.M., Carvalho, L.C.T., Dias, P.V.S., Grisi, T.C.S.L., Honorato Da Silva, F.L., Santos, S.F.M., Araújo, D.A.M.: Production and characterization of thermophilic carboxymethyl cellulase synthesized by *Bacillus* sp. growing on sugarcane bagasse in submerged fermentation. Braz. J. Chem. Eng. **32**, 35–42 (2015). https://doi.org/10.1590/0104-6632.20150321s00003298

47. Ariffin, H., Abdullah, N., Umi Kalsom, M.S., Shirai, Y., Hassan, M.: Production and characterization of cellulase by *Bacillus pumilus* EB3. Int. J. Eng. Technol. **3**, 47–53 (2006)

48. Baraldo Junior, A., Borges, D.G., Tardioli, P.W., Farinas, C.S.: Characterization of β-glucosidase produced by *Aspergillus niger* under solid-state fermentation and partially purified using MANAE-agarose. Biotechnol. Res. Int. **2014**, 1–8 (2014). https://doi.org/10.1155/2014/317092