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Establishment of Efficient Cellulolytic Bacterial Consortium Potential for Designed Composting of Rice Straw

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Abstract

The present study aims to establish "Designed Composting Technology" of rice straw by developing highly efficient cellulolytic bacterial consortium with favorable environmental conditions of maximum productivity. For this regard, one hundred and nine thermotolerant cellulose decomposing bacterial isolates were obtained from twenty natural sources collected from Egypt. Amongst them, isolates 11-AC and 81-AC were considered as the most potent isolates in term of their high cellulase productivity beside their growth at wide range of temperatures (30–65°C).These isolates were identified as *Bacillus amyloliquefaciens* 11-AC and *Bacillus licheniformis* 81-AC based on their morphological, physiological and biochemical characteristics, as well as phylogenetic analysis of 16S rDNA gene sequences. Environmental conditions affecting solid state fermentation including pH, temperature, rice straw concentration, and compost additives were evaluated. Both strains exhibited considerable cellulase productivities at wide range of pH values (5.0–9.0), temperature (30–65°C) with maximum efficiency at pH 7.0, and 55°C. In addition, 50% and 60 % (*w/v*) of rice straw achieved the maximum cellulase productivities by strains 11-AC and 81-AC, respectively. Feldspar (0.75 %, *w/v*), dolomite (0.75 %, *w/v*), rock phosphate (0.5 %, *w/v*), and zinc (0.025 %, *w/v*) were significantly enhanced bacterial growth and cellulase productivities by both strains. Finally, a consortium of strains 11-AC, 81-AC and *Bacillus sonorensis* 7-1v at 0.5 % (*v/v*) achieved a significant increase in cellulase productivity compared to single strain, mixed culture of two strains or even with commercial effective microorganisms (EM).

Keywords: Composting; rice straw utilization; *Bacillus amyloliquefaciens*; *Bacillus licheniformis;* bacterial consortium; compost additives.

1. Introduction

Rice (*Oryza sativa*) is the third most important grain crop in the world behind wheat and corn. It is widely consumed staple food for more than 60 percent of the world's human population, especially in East and South Asia, the Middle East, Latin America, and the West Indies. It is grown in at least 114 countries with global production of 645 million tons, of which about 90 % are produced in Asia (**Sharif *et al*., 2014**). Every kilogram of harvested rice is accompanied by production of about 1–1.5 kilogram of the straw (**Binod *et al*., 2010**). Therefore, rice straw is one of the abundant lignocellulosic waste materials in the world that comprise about 731 million tons produced annually [Africa, 20.9 million tons; Asia, 667.6 million tons; Europe, 3.9 million tons; and America, 37.2 million tons] (**Sarkar and Aikat, 2013**). Egypt is the largest rice producer in Africa with an approximate annual straw production of 3.1 million tons (**Abdelhady *et al*., 2014**).

The options for the disposition of rice straw materials are limited by the low bulk density and slow

degradation in the soil; therefore, these wastes are being left unutilized and can cause many problems to farmers as well as to the environment. The common manner adopted by farmers to eliminate these materials is opening ignition in fields that increase the air pollution and consequently affects public health (**Binod *et al*., 2010**). On the other hand, rice straw has several characteristics that make it a potential feedstock for production of several valuable products through microbial biodegradation processes. In terms of chemical composition, the straw predominantly contains cellulose (32–47%), hemicellulose (19–27%) and lignin (5–24%) (**Saha, 2003; Garrote *et al*., 2002**). So that, efficient utilization of rice straw *via* environmentally and friendly waste management approach including zero waste and zero burning has become quite important candidates. These approaches emphasize minimal waste, re-use and recycling of farm products, composting, and utilization as energy source, or re-use for landscaping and other many useful impacts (**Chamhuri, 2005**).

The most common practices for recycling rice straw wastes are composting process as it is the most promising low-cost technology to convert agro-industrial contaminant solid wastes into value-added organic fertilizer (**Ghosh, 2004; Tiquia and Tam, 2002; Misra *et al*., 2003**). In this process, microorganisms degrade organic matter to produce ammonia, carbon dioxide, water, heat and humus (The relatively stable organic end products). Composting has several benefits such as enhancing soil fertility, increasing agricultural productivity, improving soil biodiversity, reducing ecological risks and ameliorating the environment.

Under the optimal conditions, composting passed through three phases; mesophilic, thermophilic and cooling/maturation phases. Composting process is influenced by several factors including the particles size, feedstock utilization, the C/N ratio, nutrients amendment, pH, aeration, moisture content, temperature, and bio-accelerator (**El–Haddad *et. al*. 2014**). Traditional composting procedures take as long as several months to produce final compost product, therefore, rapid composting technology should be developed by inoculating the lignocellulosic substrates used for composting with microbial strains (compost activator) capable of a cellulose or hemicellulose decomposition.

Traditional composting is too slow for farming two or three crops per year. Therefore, it has been reported that microbial inoculants especially cellulose decomposing microbes improved composting process. **Jusoh *et al*., (2013)** reported an enhanced mineralization in composting of rice straw by the application of commercial effective microorganisms (EM•1®) (a combination of approximately 80 different microorganisms including mainly yeasts, actinomycetes and phototrophic and lactic acid bacteria which developed by Professor Teruo Higa in the 1970s at the Ryukyus University, Okinawa, Japan). **Lim *et al*., (2014)** also reported that inoculation of microbial additive (EM•1™) had improved the efficiency of composting process using oil palm empty fruit bunches. Another technology is "*IBS Rapid composting technology*", introduced in 1986, which is a technology generated by Dr. Virginia Cuevas –a professor from the University of the Philippines. This method speeds up the composting process by inoculation of agricultural substrates or residues used for composting with a cellulose decomposer fungus, *Trichoderma harzianum*, **(Virginia, 1997).** However, a major disadvantage for industrial applications of fungal strains or EM is that the lack of thermal stability at high temperatures. Composting processes passed through different phases, mesophilic (20–40 °C) in which the microbes in EM work best during this phase i.e. 25 to 45°C (http://www.golden-mist.net/en/em.php) while their activities were minimized or ceased at thermophilic phase (40–60 °C) **(Liu *et al*., 2011)**. Therefore, it is necessary to establish thermotolerant/thermophilic microbial consortium having good stability at high temperature.

Furthermore, composting process using cellulolytic thermophilic bacterial strains as an accelerator to speed up the composting process and increase nutrients in the compost has not been well developed yet. **Abdel-Rahman *et al*., (2016)** reported an efficient composting process by inoculating rice straw materials with mixed culture of *Bacillus* spp. that reduced the composting time by 40–43%. However, their composting process was lasted for 51–58 day. Therefore, the aim of this study was the establishment of new method named "Designed Composting Technology (DCT)" that involved: the development of efficient thermotolerant and cellulolytic bacterial consortium, and the optimization of compost additives (such as feldspar, dolomite, rock phosphate, and zinc) that provide favorable conditions to accelerate the decomposition of agricultural wastes. In order to establish this technology, isolation, characterization, and identification of thermotolerant cellulolytic bacteria capable of decomposing rice straw wastes were investigated. The influence of culture conditions

and compost additives on cellulase productivity by the most potent bacterial isolates were evaluated. A comparison between the developed bacterial consortium and the commercial effective microorganisms EM• 1® was also conducted.

2. Materials and Methods

2.1. Isolation of cellulolytic bacteria

Twenty environmental sources (50 samples) which used for isolation of cellulose degrading bacteria were collected from different localities in Egypt. These samples were compost materials, fertile soil, fresh cattle dung, and animal and birds fecal samples.

One gram of each sample was diluted in sterile water (1:100, *w/v*). Then, 1 mL was added into 99 mL enrichment medium which contained (per liter) 10.0 g of cellulose, 2.0 g of $NaNO_3$, 1.0 g of K_2HPO_4 (anhydrous basis), 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4.7H_2O$, and 1.0 g of yeast extract. The initial pH value was adjusted to 7.0 with 5 N NaOH or 5 N HCl. After 3-day incubation at 45 °C, 0.1 mL of broth cultures were then transferred to agar plates comprising the same components of enrichment cultural media, and incubated for 3 days at 45°C. The obtained isolates were then selected and re-cultured on agar plates by streaking methods. The bacterial purification were confirmed by using triplicates of plating to ensure the purity of the isolated colonies.

Qualitative screening for cellulase productivity were tested for all obtained isolates on solid medium containing 10 g/l of microcrystalline cellulose [Avicel, PH101, Alpha chemika] as a sole carbon source at different temperatures ranged 30–65°C and incubated for 72 h. To determine the cellulase activities, diameters of clear zones (cm) around each colony were visualized by flooding agar plates with Gram's iodine solution. While quantitative screening was examined by using mineral medium with the same components mentioned before and supplemented by 2% rice straw instead of cellulose and incubated at 45°C for 72 h. The most potent isolates were selected on the basis of the highest cellulase activities.

2.2. Enzyme Assay

Total cellulase productivity was determined by measuring the amount of reducing sugar formed from filter paper according to the methods recommended by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology (**Ghose, 1987**). Filter paperase (FPase) activity was determined by incubating 0.5 mL of supernatant with 1.0 mL of 0.05 M phosphate buffer (pH 7.0) containing Whatman no.1 filter paper strip–1.0 × 6.0 cm (=50 mg). After incubation for one hour at 50°C, the reaction was terminated by adding 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of reaction mixture. The tubes were then boiled at 100°C for 5 minutes in water bath. Reducing sugars were estimated spectrophotometrically with 3,5-dinitrosalicylic acid as described by **Miller, (1959**) using glucose as standards. The enzymatic activity was defined as an international units (IU). One unit is defined as the amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per mL per minute.

2.3. Characterization and identification of the most potent isolates

2.3.1 Morphological and biochemical properties

Morphological characteristics including shape, arrangement, Gram's reaction, and spore formation of the most potent isolates were investigated. Physiological and biochemical characterizations were performed using classical methods according to **Bergey's Manual of Determinative Bacteriology (2005)** and API identification kits [API 20E and API CHB 50 (Biomerieux, France)] (**Logan and Berkeley, 1984**). These tests include: catalase test, oxidase, methyl red test, citrate utilization test, urease, nitrate reduction, H_2S production, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, acetoin production, gelatinase, amylase, and sugar fermentation tests.

2.3.2 16S rRNA sequencing and phylogeny analyses of bacterial isolates

For 16S rRNA gene amplification, genomic DNA of bacterial isolates 11-AC and 81-AC were extracted according to the modified method by **Miller *et al.*, (1999**) and used as templates for PCR amplification. Briefly, individual colonies from agar plate were picked up using a sterile toothpick and re-suspended in 50 µl sterile deionized water. Cell suspension was cooked for 10 minutes in a water bath at 97°C, then cell lysate was centrifuged at 15,000 ***x*** g for 10 minutes. The supernatant containing the DNA was removed and an aliquot (1 µl) was added to a PCR Master Mix (Fermentas). Following this: 16S rRNA was amplified in polymerase chain reaction (PCR)

using the genomic DNA as template and bacterial universal primers of 27 f (5-GAGTTTGATCACTGGCTCAG-3) and 1492 r (5-TACGGCTACCTTGTTACGACTT-3) to amplify an approximately 1500 pb of 16S rRNA gene.

The PCR mixture (50 µl) contained 1×PCR buffer, 0.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (QIAGEN), 0.25 mM dNTP, 0.5 µM of each primer, and 1µl of extracted bacterial genomic DNA. The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) with a hot starting performed at 94°C for 3 min, followed by 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min, followed by a final extension performed at 72°C for 10 min.

The PCR products were sequenced at the GATC Biotech Company by use ABI 3730xl DNA sequencer. The 16S rRNA sequence was compared against the GenBank database using the NCBI BLAST program. Sequences were then compared with 16S rRNA sequences in the GenBank database using BLASTN (blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their identity. Multiple sequence alignment was done using ClustalX 1.8 software package (http://wwwigbmc.u-strasbg.fr/BioInfo/clustalx) and a phylogenetic tree was constructed by the neighbor joining method using MEGA (Version 6.1) software. The level of each branch (1,000 repeats) was tested by bootstrap analysis.

2.4. Solid stat fermentations

2.4.1 Pre-culture (inoculum) preparations

Single colonies of the most potent isolates were cultivated into a pre-culture medium that consisted of mineral salt medium (same component as enrichment culture) containing 1% avicel and incubated for 24 h at 50 °C and then inoculated at 5.0 % into the main fermentation media.

2.4.2 Preparation of effective microorganisms activated solution (EMAS)

The commercial EM (EM•1®) was used in this study. EMAS is an activated EM suspension in a mixture of molasses (sugar cane) and non-chlorinated water. For the activation of EM• 1®, one part EM• 1® microbial inoculants and one part of molasses were mixed with 20 parts of chlorine-free water. This solution was then stored for three to seven days in air tight expandable container for fermentation. Built up gas was released once daily. It was ready for use when it gave sweet and sour smell after a week. The pH of the EMAS was recorded to be around pH 3.5 to 3.7.

2.4.3 Main fermentations

The main fermentations were conducted in 100 mL-flasks using mineral salt medium (containing same components as enrichment culture) supplemented with rice straw (40%, *w/v*) as a sole carbon source unless otherwise mentioned. pH was adjusted at 7.0. Sterilization was carried out by autoclaving for 20 min at 121°C. Each flask was inoculated with different microbial isolates at 5 % (*v/v*) and incubated at 45°C (unless otherwise mentioned) for 48 h, then cellulase activities (FPase) were investigated as mentioned above.

2.5 Factors affecting solid state fermentation from rice straw by the most potent strains

2.5.1 Effect of initial pH values

Mineral medium contacting rice straw (40%, *w/v*) was prepared in 100-ml flasks. Each flask contains 4 g of dry rice straw and 10-mL of mineral medium. The initial pH values were adjusted at 5.0, 6.0, 7.0, 8.0 and 9.0 with 1N HCL and 1N NaOH using pH meter. The flasks were inoculated at 5.0% from the pre-culture and then incubated at 45°C for 48 h. At the end of incubation, 10 ml of distilled water was added to each flask and mixed well. Then, enzymes ware extracted by filtration. The filtrate was centrifuged at 6000 rpm under cooling condition (4°C) for 10 min. The enzymatic activities were then estimated (IU/g-dry rice straw) to determine the optimal pH value.

2.5.2 Effect of different incubation temperatures

Rice straw (40%, *w/v*) mineral medium was prepared in 100-ml flasks as described above, the pH was adjusted at 7.0. The flasks were inoculated at 5.0% of pre-culture and then incubated at different temperatures *viz*, 30, 40, 45, 50, 55, 60 and 65°C for 48 h. Cellulases were then extracted and assayed as described before to determine the optimal temperature value.

2.5.3 Effect of different concentrations of rice straw

Different concentrations of rice straw *viz.*, 40, 50 and 60 % (*w/v*) were supplemented into mineral salt media in 100 ml Flasks. Media were inoculated by bacterial strain under investigation at 5.0%. The flasks were then incubated at 55°C for 48 h. At the end of incubation, 10 mL of distilled water was added to each

flask, mixed well, cellulases were extracted by filtration. Filter paperase activities were determined (IU/g-dry rice straw) as described previously.

2.5.4 Effect of compost additives

Eight compost additives were provided from Al-Ahram mining company for organic fertilizers, Cairo, Egypt and supplemented to the fermentation medium at various concentrations. Rock phosphate (18% P_2O_5), Feldspar (12% K_2O), and dolomite (MgO) were applied at 0.75% (*w/v*), Natural iron powder, zinc and manganese salts were supplemented at 0.050% (*w/v*), whereas gypsum or lime were supplemented at 5.0% (*w/v*) of the fermentation media. All optimal conditions that recorded before were taken into consideration. At the end of incubation periods, cellulase productivities were measured as previously mentioned.

To determine the best concentration of the cellulase activator, the additives were tested at different concentrations. Rock phosphate, Feldspar, and dolomite were applied at 0.5, 0.75 and 1.0 % (*w/v*) of the production medium whereas zinc powder was applied at 0.025, 0.050 and 0.075% (*w/v*). The chemical composition of these additives were analyzed at central laboratories sector, Egyptian mineral resources authority (EMRA), Ministry of Petroleum of Egypt as shown in **Table (1)**.

Table 1: Chemical composition of compost additives (Rock phosphate, feldspar, dolomite, and zinc powder)

Component (%)	Rock Phosphate	Feldspar	Dolomite	Zinc (Zn)
SiO_2	11.7–14.6	70.9 – 71.4	0.88–1.2	17.4
TiO_2	0.03–0.08	0.02–0.02	0.03–0.03	0.14
Al_2O_3	0.59 – 1.15	14.2–14.6	0.13–0.25	5.37
Fe_2O_3	1.35 – 1.74	0.27–0.30	0.18– 0.42	**14.5**
MnO	0.05– 0.14	<0.01 – 0.01	<0.01–<0.01	1.48
MgO	1.39–2.78	<0.01–<0.01	**15.3–17.8**	1.26
CaO	**43.2 – 43.6**	<0.01– 0.30	**31.6–32.5**	**12.5**
Na_2O	0.28–0.35	1.36 –1.65	0.2–0.49	< 0.10
K_2O	0.05–0.18	**10.1–12.1**	0.06–0.08	0.35
SO_3	1.80–4.15	–	–	20.5
P_2O_5	**18.2–22.5**	<0.01– 0.09	–	0.14
ZnO	–	–	–	**14.2**

2.5.5. Effect of different inocula sizes using single strain, co-cultures and EM

The strains obtained in this study (*Bacillus amyloliquefaciens* 11-AC and *Bacillus licheniformis* 81-AC) and *Bacillus sonorensis* 7-1v (**Abdel-Rahman *et al.*, 2015b**) were pre-cultured separately for 24 h. The growth of these strains were adjusted at OD 1.0. To study the effect of mixed culture, bacterial pre-cultures were firstly prepared by mixing two or 3 strains (1:1 or 1:1:1) before inoculation to the main production medium. Single strain or mixed culture and Effective microorganisms (EM) were used as an inoculum at 0.5, 1.0, 2.0, 3.0, and 5.0% (*v/v*) of the production medium that contained mineral medium supplemented with feldspar (0.75%, *w/v*), dolomite (0.75%, *w/v*), rock phosphate (0. 5%, *w/v*), and zinc (0.025%, *w/v*) under all optimal conditions obtained. At the end of incubation periods, the activity of cellulase enzyme were measured as previously mentioned.

2.6. Statistical analysis

Data were statistically analyzed by SPSS v17, one-way and two way analyses of variance (ANOVA) tests were used for multiple sample comparison, when normality and homogeneity of variance were satisfied, followed by multiple comparison Tukey test.

3. Results and Discussion

3.1 Isolation and screening of cellulose-degrading bacteria

Fifty samples from different natural sources collected from different localities in Egypt were used for isolation of cellulytic bacteria at 45°C. Totally, one hundred and nine morphologically distinct isolates were obtained and purified. The purified bacterial isolates were subjected to celluolytic activity by growing on cellulose agar medium at 45 °C for 72h. Among these isolates, 26 isolates showed the highest cellulolytic activity on cellulose agar medium (clear zones around the bacterial colonies were greater than 0.5 cm).

Those isolates were further screened for cellulase production at different temperatures (30–65°C). All of these isolates exhibited cellulase productivities at 30–50°C. On the other hand, 24 isolates, 17 isolates, and 5 isolates exhibited cellulase productivities at 55°C, 60°C, and 65°C, respectively **(Fig. 1)**. The results showed that isolates 11-AC, 51-BA, 81-AC, 8-AA, and 99-A were exhibited the highest cellulase production in term of the zone of clearance around bacterial colony at all tested temperature values (30–65°C) that ranged 3.5–4.6 cm, 2.0–4.3 cm, 2.7–4.9 cm, 0.2–2.3 cm, and 0.4–3.1 cm, respectively. Amongst those, isolates 11-AC and 81-AC showed the highest cellulase productivities **(Fig. 2 A, B)**.

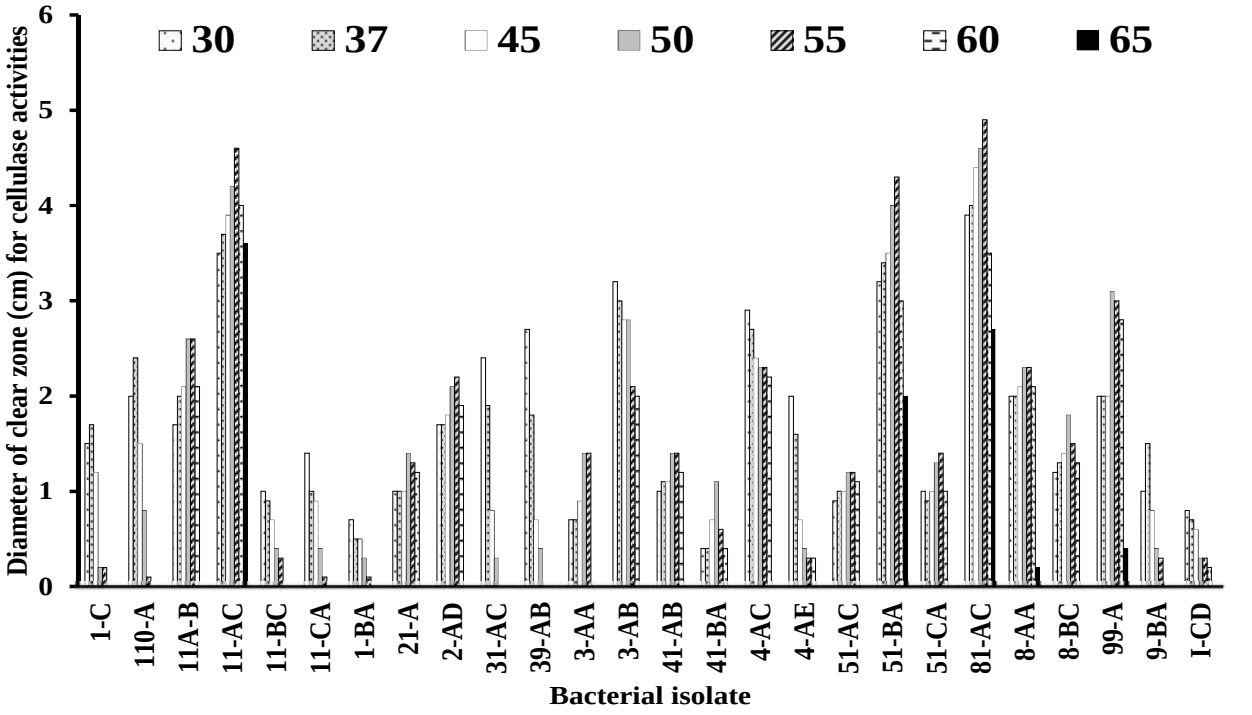

Fig.1. Cellulase productivities in term of zone of clearance (cm) for the most potent 26 bacterial isolates at different temperatures (30-65°C).

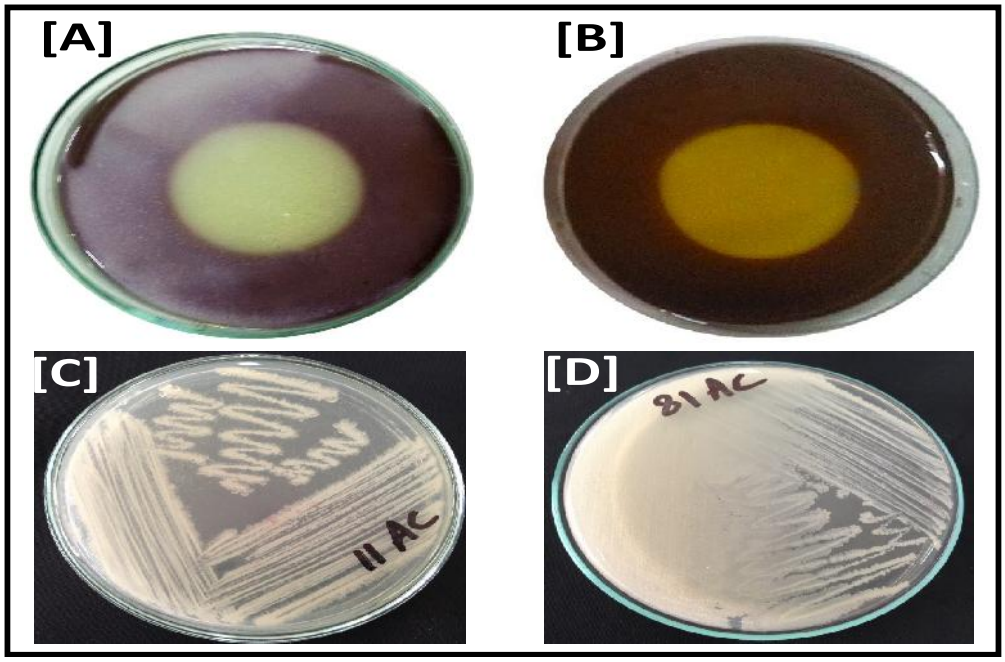

Fig.2. Cellulase productivities in term of zone of clearance around microbial growth for isolate 11-AC [A] and isolate 81-AC [B]. Culture characteristics of isolate 11-AC [C] and isolate 81-AC [D] on nutrient agar plates.

The best 26 isolates bacterial isolates were further screened for quantitative analysis of cellulase production using batch fermentation by growing in mineral medium containing 2.0% rice straw as a sole source of carbon at initial pH 7.0, 45 °C for 72 h. The highest exoglucanase (avicelase), endoglucanase (CMCase), and total cellulase (Filter paperase, FPase) activities [U/g-dry rice straw] were recorded for the isolates (Data not shown). All isolates exhibited rice straw degradation with varied cellulase productivities. Whereas, the highest cellulase productivities were exhibited by isolates 11-AC and 81-AC and therefore they were selected as the most potent isolates for further studies.

3.2 Identification of the most potent bacterial isolates

3.2.1 Morphological and biochemical characterization

The selected isolates (11-AC and 81-AC) were identified depending on their morphological **(Fig. 2 C, D)** and biochemical properties based on Bergey's Manual of systematic Bacteriology 2nd ed. (vol. 3: The Firmicutes) according to **Niall and Paul (2009).** The tested isolates were Gram positive, rod shaped, endo spore-forming bacteria, aerobic and positive for catalase and oxidase, starch hydrolysis, gelatin hydrolysis, citrate utilization and ornithine carboxylase, and gave negative results for methyl red test and H_2S production. The details of biochemical characteristics of cellulase producing isolates are shown in **Table (2).**

Table 2: Morphological characteristics and biochemical properties of isolates 11-AC and 81-AC.

Characteristics	Isolate 11-AC	Isolate 81-AC
Morphological		
Shape	Rod	Rod
Gram reaction	+	+
Spore formation	+	+
Biochemical		
Catalase	+	+
Oxidase	+	+
Methyl red	–	–
Citrate utilization	+	+
Urease	–	+
Nitrate reduction	–	+
H_2S production	–	–
β-galactosidase	–	+
Arginine dihydrolase	–	+
Lysine decarboxylase	–	+
Ornithine decarboxylase	+	+
Tryptophane deaminase	–	+
Acetoin production	–	+
Gelatinase (Gelatin hydrolysis)	+	+
Amylase (starch hydrolysis)	+	+

The sugar fermentations results using API 50 showed that, both isolates utilized various sugars including glycerol, D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, D-sorbitol, methyl-alpha-D-glucopyranoside, amygdalin, D-cellobiose, D-maltose, D-saccharose and glycogen **(Table 3)**. Most of these sugars are lignocellulose-derived sugars. The preliminary characteristics suggested that 11-AC and 81-AC were *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, respectively.

Table 3: Results of sugar fermentation test (API 50 CHB) for isolates 11-AC and 81-AC after 48 h.

No.	Sugar	Isolate 11-AC	Isolate 81-AC	No.	Sugar	Isolate 11-AC	Isolate 81-AC
1	Glycerol	+	+	26	Salicin	–	+/–
2	Erythritol	–	–	27	D-cellobiose	+	+
3	D-Arabinose	–	–	28	D-Maltose	+	+
4	L-Arabinose	+	–	29	D-Lactose	–	–
5	D-Ribose	+	+	30	D-Melibiose	–	–
6	D-Xylose	+	+	31	D-Saccharose	+	+
7	L-Xylose	–	–	32	D-Trehalose	–	–
8	D-Adonitol	–	–	33	Inulin	–	–
9	Methyl-ß- D -xylopyranoside	–	–	34	D-Melezitose	–	–
10	D-Galactose	+	–	35	D-Raffinose	–	–
11	D-Glucose	+	+	36	Amidon	–	–
12	D-Fructose	+	+	37	Glycogen	+	+
13	D-Mannose	+	+	38	Xylitol	–	–
14	L-Sorbose	–	–	39	Gentiobiose	+	–
15	L-Rhamnose	–	+	40	D-Turanose	–	–
16	Dulcitol	–	–	41	D-Lyxose	–	–
17	Inositol	+	–	42	D-Tagatose	–	+
18	D-Mannitol	–	+/–	43	D-Fucose	–	–
19	D-Sorbitol	+	+	44	L-Fucose	–	–
20	Methyl - α -D-mannopyranoside	–	–	45	D-Arabitol	–	–
21	Methyl- α D-glucopyranoside	+	+	46	L-Arabitol	–	–
22	N-acetyl glucosamine	–	–	47	Potassium gluconate	–	–
23	Amygdalin	+	+	48	Potassium 2-ketogluconate	–	–
24	Arbutin	–	+	49	Potassium 5-ketogluconate	–	–
25	Esculin	+	+				

3.2.2 Molecular identification

The results of PCR amplification of 16S rRNA gene showed that, single bands with the expected size of 1500 bp were observed. The identification was confirmed by sequence analysis of 16S rDNA gene fragments. Sequence and BLAST analyses against 16S rRNA bacterial data base of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) in Gene Bank showed that, isolates of 11-AC and 81-AC were had similarity of 99% to *Bacillus amyloliquefaciens* strains (accession numbers of KR780369.1, CP006960.1, KR259186.1 and KT185098.1) and *Bacillus licheniformis* strains (accession numbers of CP014781.1, LC127257.1, KT720050.1, CP012110.1, KJ872525.1 and KM226905.1), respectively.

The phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed using neighbor joining (NJ) a distance-based algorithm of phylogenetic analysis. Bacterial isolates (11-AC and 81-AC) were clustered **(Fig. 3)**. Both isolates showed the topology of *Bacillus* sp. The sequence of 11-AC was most closely related to *B. amyloliquefaciens* and the sequence of 81-AC was most closely related to *B. licheniformis*.

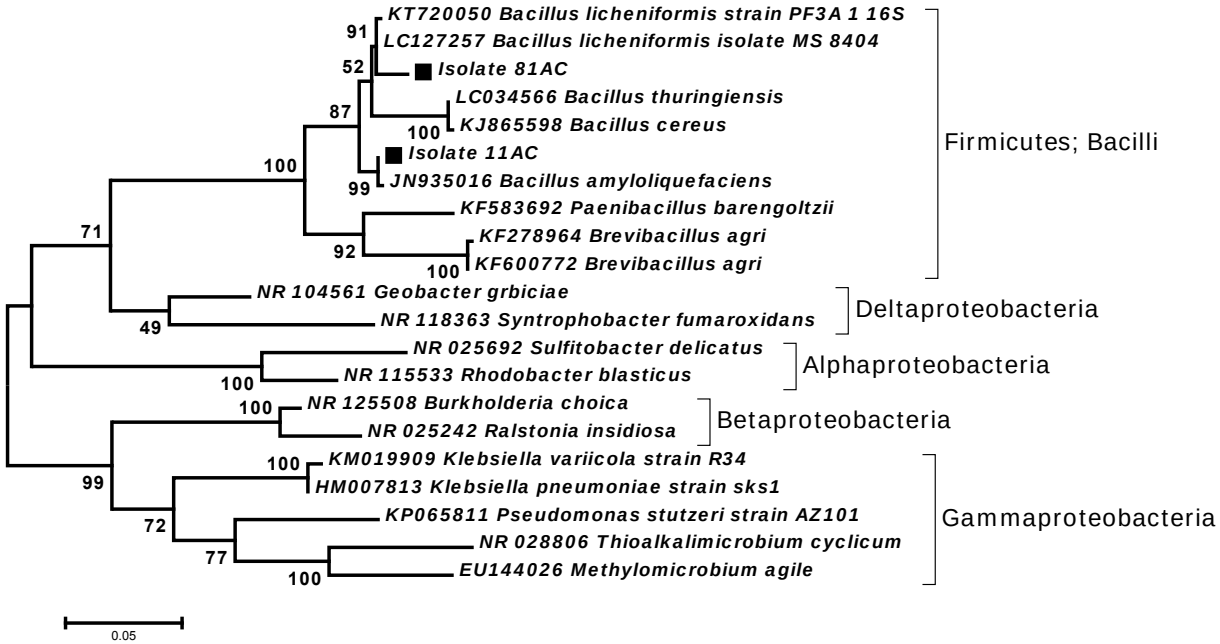

Fig. 3. Phylogenetic analysis of 16S rRNA sequences of the bacterial isolates (11-AC and 81-AC) with the sequences from NCBI. Symbol ■ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using neighbor-joining method.

Several cellulolytic bacteria have been isolated from different environmental habitats, such as the compost materials (**Abdel-Rahman *et al*., 2015a, b**), different soils (**Soares *et al*., 2012**), organic wastes (**Eida *et al*., 2012**), and ruminant animal wastes (**Maki *et al*., 2009, Singh *et al*., 2013**). Most of bacterial isolates have been identified as *Bacillus* including *B. stearothermophilus* and *B. licheniformis* (**Hala and Priest, 1994**); *B. amyloliquefaciens* SS35 (**Singh *et al*., 2013**), *B. subtilis* (**Kim *et al*., 2012**), *B. licheniformis* (**Abdel-Rahman *et al*., 2015a**), *B. sonorensis* (**Abdel-Rahman *et al*., 2015b**).

3.3. Effect of initial pH value

The initial pH of the fermentation medium is one of the most important environmental parameters affecting cell growth, and enzyme productivities (**Bacha *et al*., 2015**). Interestingly, even at acidic or alkaline pH values both strains produced a considerable amount of cellulase. As shown in **Fig. (4)**, *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC achieved the highest cellulase production at pH 7.0 with 36.4 and 39.8 IU/g-dry rice straw, respectively. Higher or lower pH values resulted in decreased cellulase productivity.

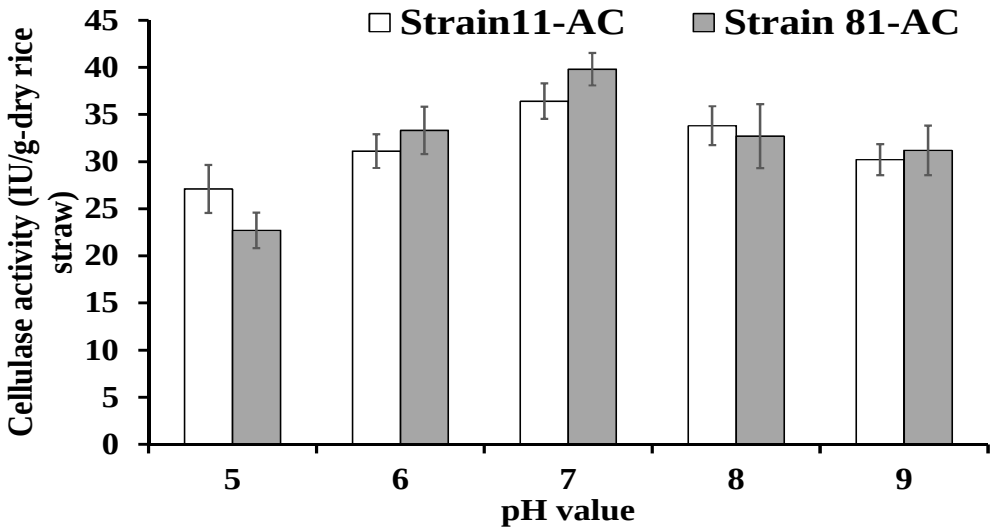

Fig. 4. Effect of initial pH value on cellulase productivity (IU/g-dry rice straw) by strain *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC under solid state fermentation using mineral media supplemented with 40% rice straw at 45°C and incubated for 48 h. Error bars are means ± SD (n=3).

These results indicated the potentiality of selected strains for rice straw decomposition even under the wide range of pH value during composting process that resulted from different fermentation products by the compost microflora. **Krishna (1999)** reported optimal pH of 7.0 for *B. subtilus* using banana fruit stalk-containing medium. These results are also in agreement with those of **Immanuel *et al*., (2006)** who found the cellulolytic enzyme obtained from *Bacillus* in the pH range of 4.0 to 9.0, with maximum productivity at pH 7.0. **Ray *et al*., (2007)** reported that pH 7.0–7.5 was more suitable for optimization of cellulase production by *B. subtilis* and *B. circulans*.

On the other hand, **Abdel-Rahman *et al*., (2015a, b)** reported the optimum pH for *B. licheniformis* 1-1v and *B. sonorensis* 7-1v were 6.0 and 5.0 with production of 43.50 and 35.0 IU/g-dry rice straw under solid state fermentation, respectively. **Karim *et al*., (2015)** reported the maximum cellulase productivity by *B. licheniformis* KIBGE-IB2 at pH 6.0 when growing in liquid fermentation medium containing CMC, wheat bran or orange peel. **Mawadza *et al*., (2000)** reported that optimal pH values for cellulases production by two *Bacillus* strains, CH43 and HR68, were in the range of 5.0–6.5. **Ou *et al*., (2009)** found that the optimum cellulase production in solid state fermentation by *B. coagulans* strain 36D1was at pH of 5.0.

3.4. Different incubation temperatures

Temperature is an important factor influences the growth of the microorganism, and enzyme production and activity **(Immanuel *et al*., 2006; Pandey, 2003).** Optimal temperatures for cellulase production are varied with different strains **(Sadhu *et al*., 2013)**. To characterize the selected stains for cellulase productivity in relation to temperature, fermentations were carried out at different temperatures (30–65°C), at pH 7 and 40 % (*w/v*) of rice straw substrate. As illustrated in **Fig. (5)**, strains 11-AC and 81-AC showed the highest enzyme production (63.8 and 70.1 IU/g-dry rice straw, respectively) and consequently high rice straw decomposition was achieved at 55°C compared to other tested temperatures. In addition, even at higher temperatures, 60–65°C both strains exhibited cellulase production that ranged 38.4–57.5 IU/g-dry rice straw. Lower decomposition of rice straw was achieved at temperatures ≤ 50 °C with the lowest cellulase productivities of 25.1 U/g-dry rice straw at 30 °C by *B. licheniformis* 81-AC.

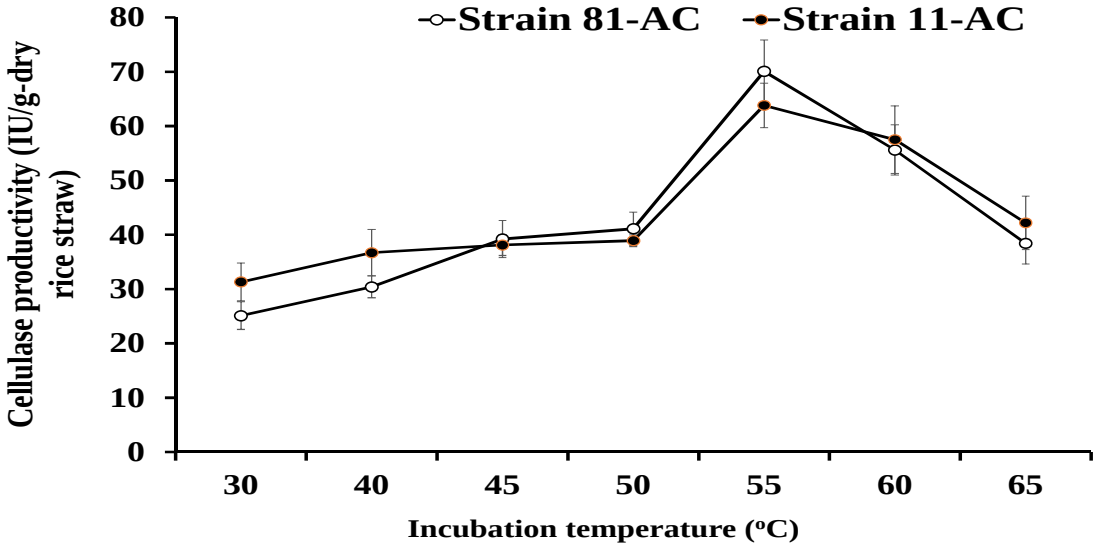

Fig. 5. Effect of incubation temperature on cellulase productivity (IU/g-dry rice straw) by strain *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC under solid state fermentation using mineral media supplemented with 40% rice straw at initial pH 7.0 and incubated for 48 h. Error bars are means ± SD (n=3).

Since composting process is proceed through three different temperatures phases: mesophilic, thermophilic and second mesophilic phases **(Abdel-Rahman *et al*., 2016)**, the present data support the utilization of selected strains for composting industry due to the high activity of the strains at various stages of compost production.

The obtained data are more related to several authors. **Abdel-Rahman *et al*., (2015 a, b)** reported that the optimum incubation temperature for maximum cellulase activity by *B. licheniformis* 1-1v and *B. sonorensis* 7-1v were 50°C and 55°C, respectively. **Acharya and Chaudhary (2012)** reported an optimal temperature of 50–55°C for cellulase production by

B. licheniformis MVS1 *and Bacillus* sp. Also, the optimal temperature for cellulase production by *B. carboniphilus* CAS 3 **(Annamalai *et al.*, 2014),** and *B. coagulans* strain 36D1 **(Ou *et al.*, 2009)** was 50°C.

Acharya and Chaudhary, (2011) reported an optimal temperatures of 65°C for cellulase production by *B. licheniformis* and *Bacillus* sp. *Bacillus* strains, CH43 and HR68 achieved the highest cellulase productivities at 65°C and 70°C **(Mawadza *et al.*, 2000)**. On the other hand, mesophilic ranges (30–37°C) was reported as optimal fermentation conditions by *Bacillus* species by several researchers. **Bai *et al.*, (2012)** found that the optimum temperature for better cellulase production by *B. subtilis* was 30°C. In addition, **Karim *et al.*, (2015)** reported that *B. licheniformis* KIBGE-IB2 achieved maximum enzyme production at 37°C that may retard its use for composting. Similarly **Sadhu *et al.*, (2013)** reported an optimal fermentation temperature for cellulase biosynthesis by *Bacillus* sp. strain was 37˚C.

3.5. Effect of rice straw concentrations

In order to demonstrate the effects of rice straw (substrate) concentration in solid state fermentation for high cellulase productivity, fermentation assays were conducted using three levels of rice straw concentrations (ca. 40, 50, 60 %, *w/v*) at 55°C and pH 7.0 as shown in **Fig. (6)**. Cellulase production was increased with an increase of rice straw concentration achieving the highest cellulase production at 50% (*w/v*) with production of 76.7 IU/g-dry rice straw by *B. amyloliquefaciens* 11-AC and 60 % (*w/v*) with production of 95.8 IU/g dry rice straw by *B. licheniformis* 81-AC.

Abdel-Rahman *et al.*, (2015 a, b) have reported the highest cellulase production of 41.3 and 31.2 U/g-dry rice straw at 50 % (*w/v*) rice straw in mineral salt medium by *B. licheniformis* 1-1v and *B. sonorensis* 7-1v , respectively.

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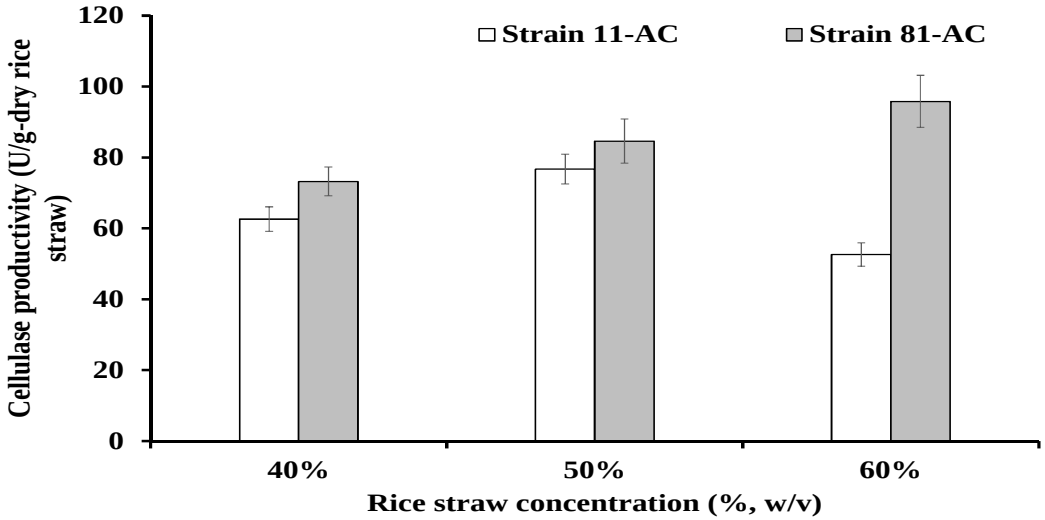

Fig. 6. Effect of rice straw concentration on cellulase productivity (IU/g-dry rice straw) by strain *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC under solid state fermentation using mineral media at initial pH 7.0, 55°C and incubated for 48 h. Error bars are means ± SD (n=3).

It has been reported that 5.0 % rice hull achieved the maximum cellulase productivity using *B. amyloliquefacince* **(Jo *et al.*, 2008). Goyal *et al.*, (2014)** obtained the maximum carboxymethyl cellulase by *Bacillus* sp. 313SI using 1% (*w/v*) pretreated rice straw. *B. licheniformis* K-3 efficiently utilized several agricultural residues (rice straw, wheat bran, wheat straw, corn waste, soybean meal, almond hulls, and mustard cake) for cellulase production at 1% **(Gupta *et al.*, 2015). Meng *et al.*, (2014)** reported that maximum cellulase productivity by *B. subtilis* BY3 was attained when cultivated in the medium containing 30 g/L corn stover.

3.6 Effect of compost additives

The influence of eight commercial natural additives (rock phosphate, dolomite, feldspar, iron, manganese, zinc, gypsum and lime) on cellulases productivities by strains 11-AC and 81-AC was investigated. The concentrations of these additives were adjusted at the recommended ranges in composting processes.

Interestingly, feldspar (10–12%, K_2O), rock phosphate (18–22%, P_2O_5), or dolomite (15–17%, MgO) at 0.75% (*w/v*) exhibited an activation of cellulase production by 25.5%, 74.1%, and 44.7% for isolate 11-AC and 40.9%, 67.6%, and 79.2% for isolate 81-AC, respectively as shown in **Fig. (7)**.

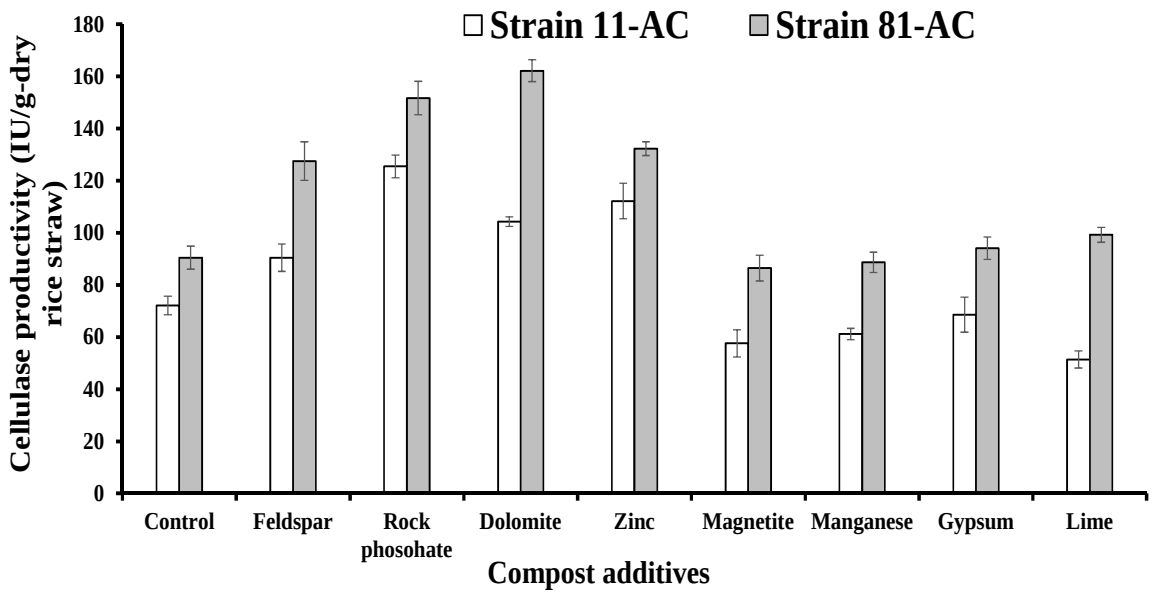

Fig. 7. Effect of various compost additives on cellulase productivity (IU/g-dry rice straw) by strain *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC under solid state fermentation using mineral media supplemented with rice straw at initial pH 7.0, 55°C and incubated for 48 h. Feldspar, rock phosphate and dolomite are supplemented at 0.75 % (*w/v*); Zinc, magnetite and manganese are supplemented at 0.05 % (*w/v*); Gypsum and lime are supplemented at 5.0 % (*w/v*). Error bars are means ± SD (n=3).

Zinc, magnetite (iron rock) and manganese were applied at 0.05% (w/v), where zinc exhibited an activation of cellulase productivity by 55.6% and 46.2% by strains 11-AC and 81-AC, respectively. On the other hand, magnetite and manganese inhibited cellulase production by 20.1%, and 15.1%, respectively for isolate 11-AC and slightly inhibited cellulase productivity at 4.4%, and 2%, respectively by isolate 81-AC. Similarly, gypsum [$CaSO_4$] and lime [$CaCO_3$] (5%, *w/v*) inhibited cellulase production by both isolates by 4.85% and 28.7%, respectively for 11-AC and by 4% and 9.6% by isolate 81-AC, respectively. This might be attributed to the change of pH values that affect bacterial growth and cellulase activity **(Gabhane *et al.*, 2012).**

Because feldspar, rock phosphate, dolomite and zinc showed cellulase activation, the effect of different concentrations of those additives on the bacterial strains was investigated during this study. Feldspar, rock phosphate, or dolomite were tested at 0.5-1.0% (*w/v*) while zinc was tested at 0.025–0.075% (*w/v*) as shown in **Fig. (8A, B)**. The highest cellulase production was achieved at 0.5 (*w/v*) rock phosphate with an activation by 80.8% and 73.5% with cellulase activity of 130.3 and 156.9 IU/g-dry rice straw for 11-AC and 81-AC, respectively. While the highest cellulase production was achieved at 0.75 (*w/v*) of dolomite and feldspar with an activation ranged 52.0–73.1% and 28.0–37.8%, respectively for both strains. The highest cellulase activation was obtained at 0.025% of zinc that achieved the enhancement by 80.5 and 63.5 % for isolate 11-AC and 81-AC, respectively. In addition, the inhibition of cellulase activities by the most of natural additives might be attributed to the presence of high concentration of heavy metals as a part in their composition as shown in **Table (1)**. From the above results, supplementation of feldspar (0.75 %, *w/v*), dolomite (0.75 %, *w/v*), rock phosphate (0. 5 %, *w/v*), and zinc (0.025 %, *w/v*) are highly recommended for effective design of composting process of rice straw using most potent strains microbial activators due to the greatest enhancement of enzymatic production and substrate degradation.

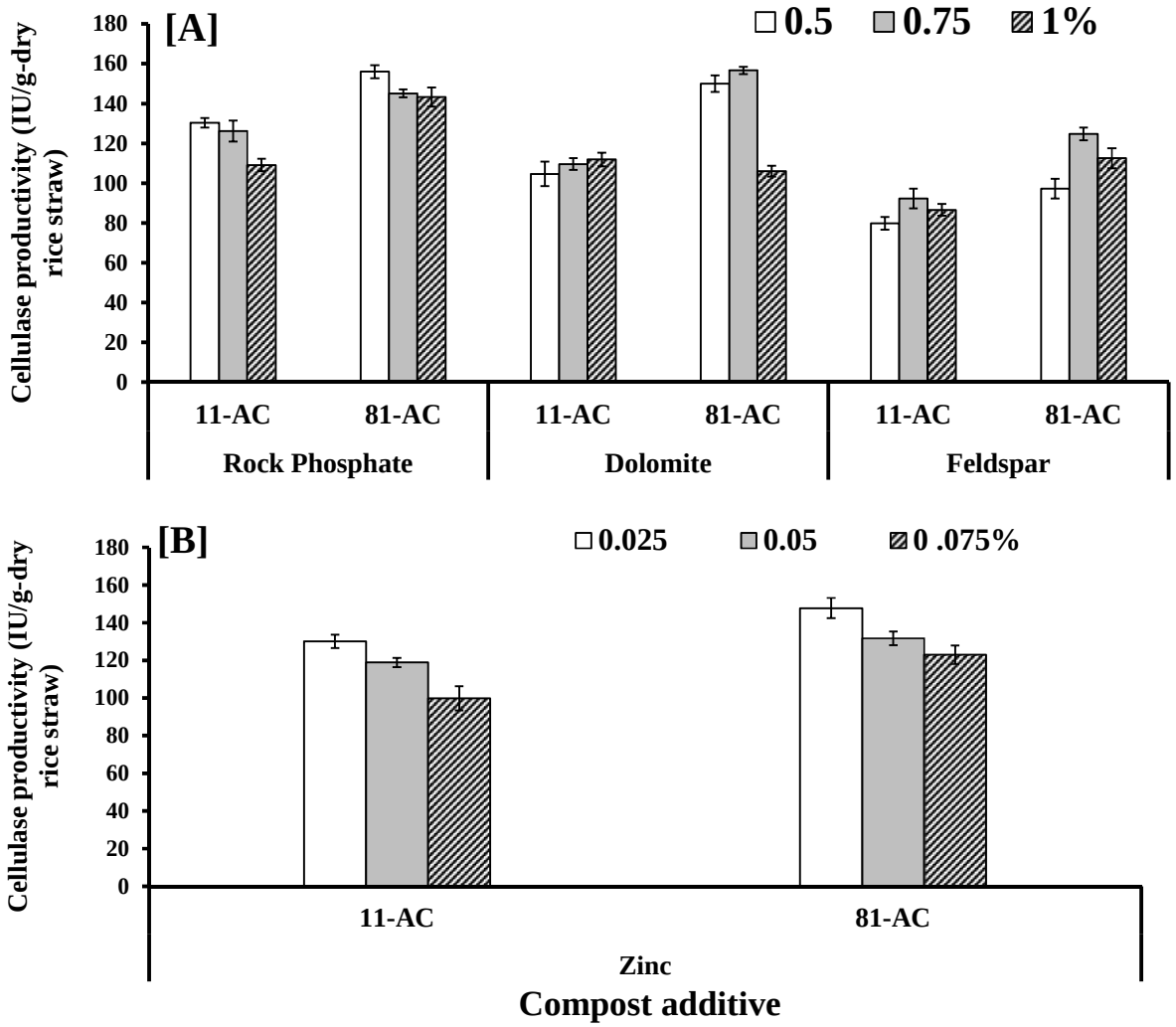

Fig. 8. Effect of different concentrations of [A] feldspar, rock phosphate and dolomite (0.5-1.0%, *w/v*) and [B] zinc (0.025-0.075 %, *w/v*) on cellulase productivity (IU/g-dry rice straw) by strain *B. amyloliquefaciens* 11-AC and *B. licheniformis* 81-AC under solid state fermentation using mineral media supplemented with rice straw at initial pH 7.0, 55°C and incubated for 48 h. Error bars are ± SD (n=3).

Metal ions were necessary for cellulase promotion by different microorganisms. **Lin *et al.*, (2012)** reported that K^+ and Mn^{2+} activated cellulase by *B. thuringiensis*. **Sreeja *et al.*, (2013)** observed that Mg^{2+} and Mn^{2+} metals enhanced cellulase production by *B. altitudinis* and *B. licheniformis*, respectively. **Abdel-Rahman *et al.*, (2015a, b)** also reported that feldspar at 0.75%, (*w/v*) has significantly enhanced cellulase production for *B. licheniformis* 1-1v and *B. sonorensis* 7-1v. Addition of magnetite (iron), or manganese salt to the fermentation medium had led to reduction of cellulase productivity by *B. licheniformis* 1-1v **(Abdel-Rahman *et al.*, 2015a)** while improved cellulase productivity by *B. sonorensis* 7-1v **(Abdel-Rahman *et al.*, 2015b). Mohee (2007)** have reported that the addition of dolomite to cellulase fermentation medium did not improve cellulase productivity. **Himanen and Hänninen (2009)** reported that found that mixture of calcium hydroxide, peroxide, and oxide had significantly positive effect on the process and quality of *in vitro* compost, while addition of sulphates and oxides of iron, magnesium, manganese, and zinc mixed with clay have negative results. Supplementation of lime to rice straw medium resulted in a decrease of cellulase production by *Bacillus* spp. 1-1v and 7-1v **(Abdel-Rahman *et al.*, 2015 a,b).** No significant influence on microbial growth and cellulase productivity had been obtained using lime, and phosphogypsum as compost additives **(Gabhane *et al.*, 2012).**

3.7. Effect of microbial inocula (single strain, mixed cultures, or EM) on the decomposition of rice straw

Previously we have reported an efficient cellulose decomposing bacterium, *B. sonorensis* 7-1v that capable of decomposing rice straw at high temperature **(Abdel-Rahman *et al.*, 2015b)**. In order to design efficient composting method for rice straw, different inoculation by single or mixed cultures of cellulolytic strains of 11-AC, 81-AC, and 7-1v were inoculated at different sizes in rice straw-media. The media were supplemented with mixed compost additives [Feldspar (0.75%, *w/v*), dolomite (0.75%, *w/v*), rock phosphate (0.5%, *w/v*), and zinc (0.025%, *w/v*)] as the best concentrations activated cellulase productivity and consequently rice straw decomposition. The obtained cellulase productivities were compared with effective microorganisms (EM•1®) under the same conditions.

Interestingly, all treatments (single and mixed culture) showed higher cellulase productivities than that obtained using EM as represented in **Table (4)**. Cellulase productivities obtained by EM was ranged 100.5–135.4 U/g-dry rice straw at all tested inocula sizes. However, cellulase production was ranged 146.1–178.8, 109.6–173.9, and 158.9–196.8 IU/g-dry rice straw by isolate 81-AC, 11-AC and 7-1v, respectively. The highest cellulase productivities were obtained using mixed culture that ranged 175.8–260.4, 195.8–292.3, 154.5–242.2, and 203.3–275.1 IU/g-dry rice straw by mixed culture of [81-AC &11-AC], [81-AC & 7.1-v], [11-AC & 7.1-v], and [81-AC &11-AC & 7.1-v], respectively. All treatments are significantly different compared to EM inoculant at all tested inoculum sizes and some treatments achieved 2–3 folds higher than those obtained with EM ($p \leq 0.05$).

Analyses of variance (ANOVA) revealed that there were significant differences and variations among the treatments to enhance the productivity of cellulase and decompose rice straw. Although inoculum size of 3% (*v/v*) of bacterial mixture of 81-AC & 7-1v achieved the highest ($F_{7,16}$ = 312.6; $p \leq 0.001$) cellulase productivity among all treatments, using smaller inoculum is recommended for decreasing labor work and composting cost. The use of 0.5% (*v/v*) from mixed cultures of 81-AC & 11-AC & 7.1-v was significantly ($F_{7,16}$ = 325.8; $p \leq 0.001$) achieved higher cellulase productivities (275.1 U/g-dry rice straw) compared to EM and other inocula (**Fig. 9**). Also, the use of 1.0% (*v/v*) of bacterial consortium (81-AC & 11-AC or 81-AC & 7-1v) exhibited a significant difference ($F_{7,16}$ = 177.8; $p \leq 0.001$) among other treatment achieving 246.4 and 234.5 U/g-dry rice straw. Also, the use of 2.0% (*v/v*) of the same consortium showed higher ($F_{7,16}$ = 165.2; $p \leq 0.001$) cellulase productivity of 247.5 and 245.5 U/g-dry rice straw, respectively.

Table 4: Cellulase productivities (IU/g-dry rice straw) by single or mixed cultures of strains 11-AC, 81-AC, and 7-1v and by EM•1® at different inocula sizes (0.5-5.0%, *v/v*) under solid state fermentation of rice straw.

Inoc. size (%, *v/v*)	Cellulase (FPase) productivity [U/g-dry rice straw]							
	Isolate 81-AC	Isolate 11-AC	Isolate 7-1v	Isolates 81-AC & 11-AC	Isolates 81-AC & 7-1 V	Isolates 11-AC & 7-1 V	Isolates 81-AC & 11-AC & 7-1 V	EM
0.5	178.8±4.35^{d}	171.4±7.41^{d}	196.8±4.82^{c}	175.8±2.13^{d}	204.5±7.20^{c}	242.2±4.18^{b}	275.1±4.46^{a}	100.5±2.65^{e}
1	166.8±4.49^{e}	173.9±7.02^{de}	185.1±4.64^{cd}	246.4±5.75^{a}	234.5±2.3^{a}	196.5±3.88^{c}	217.1±4.05^{b}	135.4±4.86^{f}
2	146.1±4.45^{ef}	171.9±5.63^{f}	181.8±2.97^{e}	247.5±8.28^{b}	254.5±7.23^{a}	195.1±10.12b^{c}	211.3±3.81^{b}	109.4±7.07^{f}
3	150.3±5.16^{ef}	134.0±7.79^{f}	158.9±7.02^{e}	258.1±3.93^{b}	292.3±9.68^{a}	179.1±4.75^{d}	217.3±4.39^{c}	116.3±3.0^{g}
5	154.8±4.38^{d}	109.6±2.29^{e}	172.4±3.84^{c}	260.5±5.85^{a}	195.8±6.85^{b}	154.5±8.27^{d}	203.3±8.44^{b}	109.3±5.03^{e}

Different letters between rows denote that mean values are significantly different ($p \leq 0.05$) by Tukey LSD test, means ± SD (n=3).

EM: Effective microorganisms.

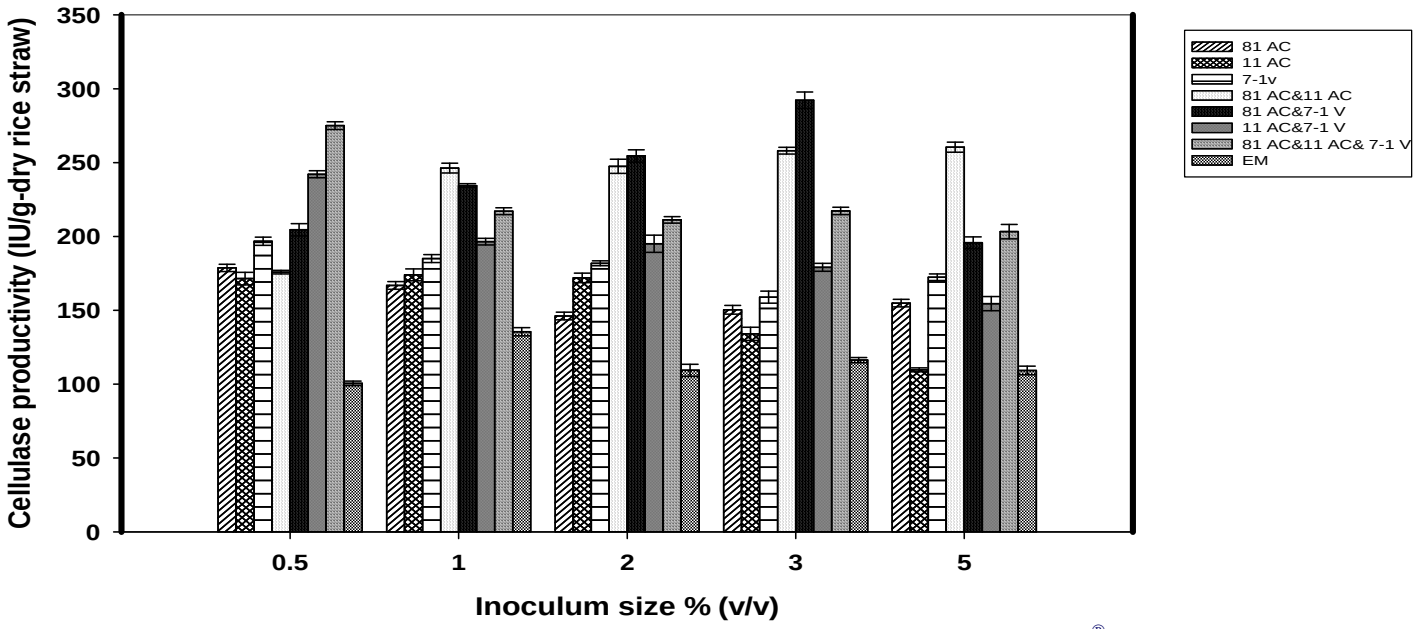

Fig. 9. Cellulase productivities (IU/g-dry rice straw) by effective microorganism (EM•1®) and single or mixed cultures of strains *B. amyloliquefaciens* 11-AC, *B. licheniformis* 81-AC, and *B. sonorensis* 7-1v at different inocula sizes (0.5-5.0%, *v/v*) under solid state fermentation using mineral media supplemented with rice straw and mixed compost additive [feldspar (0.75 %), dolomite (0.75 %), phosphate (0.5 %), and zinc (0.025 %)] at initial pH 7.0, 55°C and incubated for 48 h. Under the same inoculum size (*) denote the bacterial inoculation which caused the highest cellulase productivity ($p \geq 0.05$). Error bars are ± SE (n=3).

The present study successfully introduces innovative bacterial consortiums available for effective utilization of rice straw under specific conditions of compost components (rice straw with specific compost additive) that will be referred as Designed composting Technology "DCT" of rice straw. Further studies will be conducted for the potentiality of the established system for composting of rice straw with and without adding different animal manures; as well further investigations with different lignocellulosic waste materials will be conducted.

5. Conclusion

Amongst 109 cellulolytic bacterial isolates obtained from natural sources, isolates 11-AC and 81-AC were selected as the most potent cellulase producers and identified as *Bacillus amyloliquefaciens*11-AC and *Bacillus licheniformis* 81-AC. The parameters controlling celluase productivities under solid state fermentations using rice straw as a sole carbon source were investigated. Compost additives including feldspar (0.75 %, *w/v*), dolomite (0.75 %, *w/v*), rock phosphate (0.5 %, *w/v*), and zinc (0.025 %, *w/v*) were significantly enhanced cellulase productivities. This study successfully established new composting method named "Designed Composting Technology" of rice straw by developing efficient cellulolytic bacterial consortium that achieved significant cellulase productivities compared to commercial effective microorganisms (EM).

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