clean technologies

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Bio Ethanol Production from Rice Straw Saccharification via *Avicelase* Gene in *E. coli* Recombinant Strain

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Abstract: The most abundant organic carbon source on Earth is cellulosic materials. Its main resources are crop straws which are not commonly used and produce environmental pollution. These resources can be a site of biological hydrolysis to primary sugars by cellulase enzymes, in which avicelase is the most efficient enzyme in the cellulase family. This work aimed to clone the *avicelase* gene, transfer it to *E. coli*, optimize its expression, saccharify rice straw to its primary sugars, and ferment it to bioethanol. The *avicelase* gene was cloned from the *Bacillus subtilis* strain and cloned into two *E. coli* (i.e., DH5α and Bl21) strains. The optimized avicelase activity was described by testing the effect of different media and growth conditions including different carbon and nitrogen sources, as well as pHs and shaking or static conditions. Avicelase enzyme was extracted and used to saccharify rice straw. The obtained glucose was subjected to fermentation by *Saccharomyces cerevisiae* F.307 under an aerobic condition growth for the production of bioethanol. The ethanol yield was 5.26% (*v*/*v*), and the fermentation efficiency was 86%. This study showed the ability to clone one of the cellulolytic genes (i.e., *avicelase*) for the valorization of rice straw for producing renewable energy and bioethanol from cellulolytic wastes such as rice straw.

Keywords: rice straw; *avicelase* gene; cloning; expression optimization; bioethanol

Citation: Abdel-Salam, M.S.; Hafez, S.S.; Fadel, M.; Mohamed, S.A.H.; Hegazy, W.K.; Khalil, B.E. Bio Ethanol Production from Rice Straw Saccharification via *Avicelase* Gene in *E. coli* Recombinant Strain. *Clean Technol.* **2023**, *5*, 451–465. https://doi.org/10.3390/cleantechnol5020023

Academic Editor: Rajender S. Varma

Received: 16 January 2023
Revised: 6 March 2023
Accepted: 22 March 2023
Published: 3 April 2023

1. Introduction

Cellulose, a linear polymer made up of linked glucopyranoside units, is one of the most abundant organic carbon sources in the world and makes up a third to half of the weight of dry plants. Its main resources are crop straws, but much of it goes unused and results in waste and pollution through burying, decaying in fields, or burning [1–3]. Cellulosic substrates could be hydrolyzed by chemical and biological means. Chemical hydrolysis, although it is a rapid and efficient process, produces hazardous waste; moreover, it is energy-intensive and can inhibit the downstream processes [4,5]. On the other hand, using biological means for cellulose hydrolysis is more environmentally friendly, acts under milder conditions, and consumes lower energy [6]. However, the latter approach could be slower and dependent on the activity of used microorganisms, e.g., limiting throughput and scalability; there are also challenges in keeping consistent enzyme production [7]. Recent studies showed the promising role of biological hydrolysis in producing sugars from cellulosic substrates, including using biopreparation with mixed microbial cultures and genetically modified microorganisms, which could become an increasingly important method for producing sustainable and renewable fuels and chemicals [8,9]. The conversion of cellulose into glucose and then its utilization as an energy source for further bioconversion into useful materials has been extensively studied [10,11].

To break down cellulose completely, a group of enzymes is required [12,13]. There are three categories in the cellulase family: exo-β-1,4-glucanase (cellobiohydrolase or avicelase (EC 3.2.1.91), endo-β-1,4-glucanase (EC 3.2.1.4), and β-glucosidase (EC 3.2.1.21) [3]. They work together to break down the cellulose chain into individual sugars [1].

To increase cellulase's susceptibility to plant materials, a pretreatment process is an essential step. Using chemical and physical methods (autoclaving) enhances the degradation of both lignin and hemicelluloses [14].

Cellulase enzymes have many roles in different fields, including environmental and industrial sectors. They play a vital role in drifting the carbon cycle from various cellulose biomass [15]. They also have important applications in the textile industry [16], food, paper and pulp, laundry, waste management, and biofuel production [17,18]. The global cellulase market is estimated at USD 1621 million in 2022. The market is likely to reach nearly USD 3531.1 million by 2032, with a growing CAGR of 6.9% from 2022 to 2032 [19].

The first cellulase gene was cloned in 1982 [20], and most *cellulase* genes have been expressed in *E. coli*, *S. cerevisiae*, and *Pichia pastoris* [21–25]. The cellulase was expressed in these hosts with different activities. *Cellobiohydrlase* I (CBH I) gene was amplified from *Trichoderma koningii* genomic DNA, inserted into the pGAPZα A plasmid, then transformed into *Pichia pastoris*. The recombinant *P. pastoris* had avicelase activity in the supernatant of 0.1276 U/mL [26]. The *Cellobiohydrolase* gene was cloned from a thermophilic bacterium *Clostridium clariflavum* and expressed in *Escherichia coli* BL21 (DE3). After optimization of various parameters (pH, temperature, isopropyl b-D-1-thiogalactopyranoside (IPTG) concentration, and time of induction) the maximum enzyme activity was achieved (2.78 U mL^{-1}) [27].

Despite being a promising alternative to traditional fuels, cellulosic biofuels have not yet been produced at a cost-competitive level with current technology [26]. Using strong promoters within expression vectors is one of the promising approaches for high production levels of *cellulase* gene products and hence reduces their production cost. In another approach, by using the appropriate vector, *cellulase* genes could be expressed directly in yeast strains and acquire the cellulolytic activity to utilize cellulose and convert it to bioethanol.

To make industrial use of cellulolytic enzymes in the degradation of cellulosic materials, it is essential to research the enzymatic properties of these enzymes and understand the mechanisms behind the synergistic effects among the enzymes. As mentioned by [28], "Achieving the optimum secretion of these enzymes simultaneously remains a challenge".

Avicelase is the cellulase family's most effective enzyme, which splits cellobiosyl units from the termini of cellulose chains [29]. Typically, avicelase liberates glucose or cellobiose from the non-reducing end of microcrystalline cellulose.

Strategies for plant waste degradation concentrate on using efficient cellulolytic microorganism strains individually or in combination (biopreparation). Utilizing different cellulolytic microorganisms requires identifying the degradation conditions that best fit all used strains for the best synergistic action. The research team's overall aim is to use different cellulolytic microorganisms with different clones for efficient rice straw saccharification. For this purpose, this present study aimed to clone the *avicelase* gene and to identify its expression in different growth conditions. Additionally, this study aimed to convert rice straws into sugars through avicelase saccharification and utilized the resulting sugars to create ethanol.

2. Materials and Methods

All used chemicals are of analytical grades.

2.1. Microorganisms and Plasmids

Bacterial and yeast strains and plasmids used in this study are present in Table 1.

Table 1. Bacterial and yeast strains and plasmids used in the present study.

Code	Bacterial, Yeast Strains, Plasmids	Reference/Source
α	*E. coli* DH5α	Microbial Genetics Dept., NRC
21	*E. coli* Bl 21	Microbial Genetics Dept., NRC
BS1	*Bacillus subtilis subsp. subtilis* BTN7A	[30]
BS2	*Bacillus subtilis subsp. subtilis* 168	Bacillus Genetic Stock Center
BS3	*Bacillus subtilis* DB100	Microbial Genetics Dept. NRC
BS4	*Bacillus subtilis* Ain Shams	Faculty of Science, Ain Shams Univ.
BS5	*Bacillus subtilis* Al-Azhar	Faculty of Science, Al-Azhar Univ.
BM	*Bacillus megaterium* 7A37	Bacillus Genetic Stock Center
Alk-1	*Bacillus cereus* group isolate 1	Microbial Genetics Dept., NRC, Egypt.
Alk-2	*Bacillus cereus* group isolate 2	Microbial Genetics Dept., NRC, Egypt.
Anoxy	*Anoxybacillus flavithermus* BTN7B	[30]
Geo.	*Geobacillus stearthermophillus* 9A5	[31]
Yeast		
	Saccharomyces cerevisiae F-307	Microbial Chemistry Dept., NRC, Egypt.
Plasmid		
pGEM-Teasy	3015 bp, Amp^r	Promega Co. Madison, WI, USA
pAvi-168	pGEM-Teasy with *avicelase* gene	This study

2.2. Media

Luria–Bertani medium (LB) [32] was used for bacterial growth as it contains (g/L): NaCl, 10.0; Yeast extract, 5.0; and Tryptone, 10.0. Bushnell Haas (BHM) medium [33] contains (g/L): K_2HPO_4, 1.0: KH_2PO_4, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; and $FeCl_3.6H_2O$, 0.05. It was used for avicelase expression using 1% avicel as the sole carbon source and supplemented with 0.1 mg thiamine/100 mL medium.

2.3. Bacterium Inoculum

An equal *E. coli* inoculum was used to inoculate each medium by inoculating the corresponding amount to reach OD_{620} 0.01/20 mL from an overnight culture [30]. This inoculum contains about 1.3×10^5 CFU/mL.

2.4. Screening for Avicelase Production

A total of 11 identified strains and 54 bacterial isolates (Microbial Genetics Department, NRC, Cairo, Egypt), including 36 isolated from compost and 18 isolated from silage, were used for primary screening for avicelase production. They were inoculated on BHM medium supplemented with avicel as the sole carbon source. The positive avicelase-producing strain showed a clear zone around its growth after staining with iodine [34].

2.5. Avicelase Assay

Enzymatic assays: The activity of avicelase was assayed in triplicates by incubating 0.5 mL of the crude enzyme with 0.5 mL avicel (1.0%) prepared in 0.05M Tris-HCl buffer, pH 8.5 at 50 °C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. After 10 min of reaction, 2 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min. The resulting samples were then cooled in ice water, and the absorbance was measured at 540 nm [35]. Moreover, 1 unit (U) of avicelase activity was defined as 1 µmol of glucose as the amount of enzyme released from avicel within 1 min under reaction mixture at 50 °C.

2.6. Cloning of Avicelase Gene

All molecular biology manipulations were performed according to standard protocols [36] and kit suppliers' instructions unless specified.

PCR technique was used to amplify the avicelase gene of *B. subtilis* 168 strain, using a DNA thermal cycler (Perkin Elmer GeneAmp PCR System 9600, Waltham, MA, USA).

Different bioinformatics tools were used to select the appropriate primers. The DNA sequence of *avicelase* gene was obtained from the available data in the EMBL nucleotide sequence database of *Bacillus subtilis* 168 strain. Recombinant plasmid was mapped by using PlasMapper Version 2.0 software [37].

The *avicelase* gene sequence was analyzed for its restriction endonuclease cutting using the Webcutter 2.0 software [38]. Primers derived from *avicelase* gene sequence were obtained using Primer3 software. Table 2 represents the primer's sequences where the expected amplicon was 636 bp.

Table 2. *Avicelase* gene primers.

Code	Sequence
V-L	TTA AAG AGT ATT GTC GTA AGA CGT GA
V-R	GGG GTG GAA GTA ATG AGT GC

2.7. Optimization of Avicelase Expression

Experiments were conducted to identify the best avicelase expression conditions. *E. coli* DH5α (pAvi-168) was grown on different media, including complex medium (LB medium) and minimal medium (BHM medium) supplemented with different nitrogen sources using different shaking conditions. An equal *E. coli* inoculum was used from overnight culture. The avicelase activities were assayed. All tests were conducted in triplicates.

2.7.1. Effect of Rich and Minimal Media, and Shaking on Avicelase Activity

Avicelase activity expression of *E. coli* DH5α (pAvi-168) was studied using LB medium or BHM medium after incubation at 37 °C with shaking (120 rpm) or static conditions.

2.7.2. Effect of Inorganic Nitrogen and pH on Avicelase Activity

BHM medium was supplemented with different inorganic nitrogen (0.1%) with different pH values, inoculated with *E. coli DH5α* (pAvi-168), and incubated for 3 days at 37 °C with 120 rpm shaking; then, glucose concentrations were determined.

2.8. Utilization of Avicel or Rice Straw by E. coli Transformant

BHM medium supplemented with avicel or rice straw as the sole carbon source was inoculated with *E. coli* (pAvi-168) and *E. coli* Bl21 (pAvi-168) strains and incubated at 37 °C with 120 rpm shaking; for up to 3 days, the produced reducing sugars were monitored.

2.9. Pretreatment of Rice Straw

Crushed straws were pre-treated with 1.5% sodium hydroxide and autoclaved at 121 °C for 30 min. The alkali was then neutralized with HCl to pH 7, and the solid-treated substrates were filtered through cheese clothes, washed several times with water (until reaching neutral pH in the washed water), and, finally, dried in oven at 70 °C until constant weight was reached. Rice straw is a lignocellulosic material essentially constituted of cellulose (24.0%), hemicelluloses (27.8%), and lignin (13.5%), with important amounts of ash (17%) that correspond mostly to silica [39]. This treatment reduced crystallinity of cellulose, removing lignin to 7.6% and other inhibitors, and enhanced the subsequent process (saccharification) of rice straw cellulose [40]. This pretreatment condition yielded the highest delignification percentage of 79.6% over the lignin content in native untreated rice straw [41–43].

2.10. Rice Straw Saccharification

The recombinant strains were inoculated in LB medium and incubated at 37 °C in 120 rpm rotary shaker for 72 h. Avicelase enzyme was precipitated from supernatants using

solid ammonium sulfate at 60% (w/v) saturation with gentle stirring, then stood overnight at 4 °C. After centrifugation, the precipitate obtained by cooling centrifugation (Cooling centrifuge, HERMLE, Z 326K, Gosheim, Germany) 6000 rpm for 20 min was dissolved in 50 mM citrate buffer, pH 4.8, and dialyzed several times against fresh buffer.

The saccharification of the rice straw was performed by adding the filter sterilized–extracted enzyme to pretreatment rice straw in 50 mM citrate buffer, pH 4.8 (after sterilization by autoclaving), and incubation at 50 °C for 24 h. The liberated glucose was determined by DNS method [35].

The microbiology standard methods were followed to avoid any possible contamination, e.g., all treatments were conducted in a Class II Laminar flow cabinet, autoclaving, or bacterial-filter sterilization of all used buffers and enzymes.

2.11. Bioethanol Fermentation

The glucose syrup obtained from scarification by avicelase was concentrated by evaporation to concentrate involved sugar content of 10% (w/v). Conical flasks 250 mL capacity each contained 100 mL of YPM medium (containing (g/L) malt extract, 3; yeast extract, 3; peptone, 5; and sucrose, 30) was steam sterilized at 121 °C for 15 min, cooled to room temperature, then inoculated with a loop of yeast strain *S. cerevisiae* F-307 and incubated statically at 34 °C for 24 h. The previous yeast culture was used to inoculate the prepared fermentation vessels at 1% *v/v* [44]. The above sugar syrup was fortified with (g/L) yeast extract, 1; malt extract, 1; magnesium sulfate, 0.5; and diammonium phosphate, 2. Fifty ml was dispensed in set of 250 mL flasks after sterilization inoculated with above *Saccharomyces cerevisiae* inoculum at 1% v/v, then incubated statically at 34 °C for 72 h. Ethanol yield content of the fermented samples was measured by determination of consumed sugar after fermentation and conversion to ethanol following the equation: 1 g of glucose produces 0.51 g of ethanol, which equals $0.51 \times \frac{100}{81} = 0.62$ mL ethanol, as described in [45].

Fermentation Efficiently

Fermentation efficiently was calculated as produced ethanol divided by theoretical ethanol multiplied by 100 [45].

3. Results and Discussion

3.1. Select Efficient Avicelase Bacterial Strains

In a comparison of endoglucanase and beta glucosidase production, avicelase production was reported as scanty by a few microorganisms [46].

Small amounts of cellulolytic enzymes (less than 0.1 $g{\cdot}L^{-1}$) were produced by bacteria, while fungi produce large amounts (more than 20 $g{\cdot}L^{-1}$) of cellulases [47]; therefore, most of the research focused on studying the fungal cellulases. More efforts have to be made to isolate efficient cellulolytic bacterial strains. In this article, 65 bacterial strains were screened for their cellulolytic activities, among them *Bacillus subtilis subsp. subtilis* 168 strain has been selected for further studies.

Primary screening of avicelase-producing strains was performed by inoculating the 65 tested strains on the BHM medium supplemented with avicel as the sole carbon source resulting in selected 20 strains with high avicelase activities.

The best 10 bacterial strains were grown for 2 days in the same medium, and their avicelase-specific activities were determined (Table 3).

Table 3. Avicelase activity of tested bacterial strains.

Bacterial Strain	Specific Activity IU/mg Protein	
	24 h	**48 h**
Bacillus subtilis subsp. subtilis BTN7A	1.34	0.95
Bacillus subtilis subsp. subtilis 168	**3.29**	3.88
Bacillus subtilis DB100	0.42	0.75
Bacillus subtilis Ain Shams	0.62	1.10
Bacillus subtilis Al-Azhar	0.18	1.01
Bacillus megaterium 7A37	1.58	1.44
Bacillus cereus group isolate 1	3.43	4.74
Bacillus cereus group isolate 2	2.57	2.73
Anoxybacillus flavithermus BTN7B	2.66	2.08
Geobacillus stearthermophillus 9A5	1.5	0.59

Table 3 showed that avicelase-specific activities were raised on the second day of incubation: they range between 0.59 to 3.88. *B. subtilis* 168 strain (BS2) was among the high avicelase producers, so selected for *avicelace* gene cloning.

3.2. Avicelase Gene Cloning

Several avicelase (i.e., *Cellobiohydrlase*, *exo-β-1,4-glucanase*) genes have been cloned previously: they were different in length and properties [27,46,48–51]. The *Cellobiohydrolase* A (CBHA) gene of *Aspergillus niger* is 1500 bp [52], while it was 489 bp in *Bacillus subtilis* (GenBank: LC639863.1).

DNA was extracted from the *B. subtilis* 168 strain using a Genomic DNA Isolation kit (GeneDireX, Inc., Taoyuan, Taiwan) and used as a template for *avicelase* gene amplification. The PCR mixture was prepared including Master Mix (TIANGEN, Beijing, China). The PCR program used was 95 °C for 5 min for DNA denaturation, 30 cycles of 95 °C for 1 min, different annealing temperatures for 30 s, 72 °C for 1 min, then 72 °C for 5 min.

Gel electrophoresis was used to analyze PCR products (Figure 1). Results indicated that using 42 °C as the annealing temperature was the best in amplifying the target 636 bp DNA amplicon.

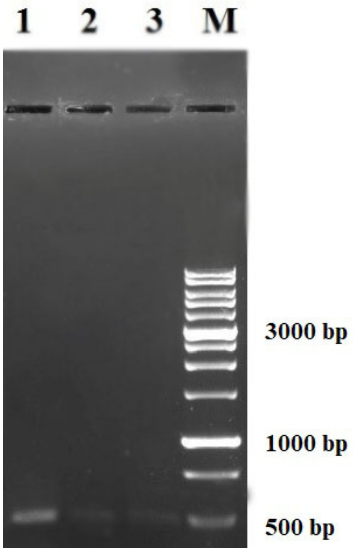

Figure 1. Agarose gel electrophoresis of PCR products. M: 1 kp DNA Ladder GeneON, lane 1; annealing 42 °C, lane 2; annealing 45 °C, lane 3; and annealing 48.8 °C.

The DNA band (636 bp) was extracted from the agarose gel using FavorPrep GEL Purification kit, (FAVORGEN, Biotech Corp., Ping Tung, Taiwan) and cloned with pGEM®-

T Easy Vector using ligation cloning kit (Promega Co. Madison, WI, USA). The obtained recombinant plasmid was named pAvi-168 (Figure 2). The recombinant plasmid was used to transform both *E. coli* DH5α and *E. coli* Bl 21 (expression host) by heat-shock treatment, and transformants were selected using ampicillin resistance and white/blue screening method (i.e., IPTG/X-gal). Different transformants were successfully obtained from both *E. coli* strains.

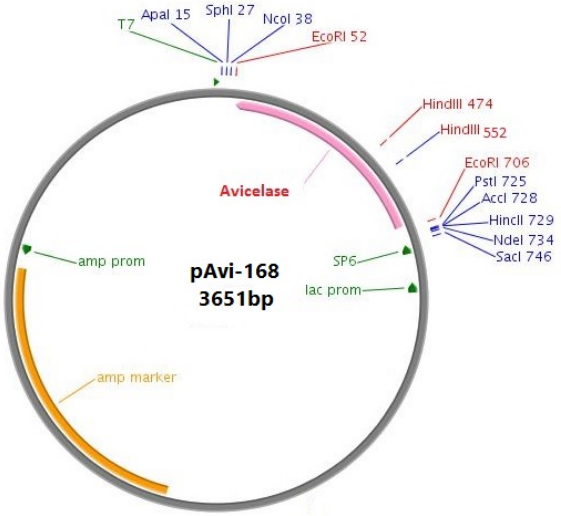

Figure 2. Avicelase recombinant plasmid pAvi-168.

Plasmids were isolated from random *E. coli* transformants and analyzed by agarose gel electrophoresis. Figure 3 represents the obtained plasmids from seven *E. coli* DH5α (pAvi-168). Results confirmed the existence of plasmid DNA bands in all tested *E. coli* transformants. The existence of different DNA bands in each tested transformant represents the different plasmid DNA forms of one plasmid, such as supercoiled, open circular, or linear plasmid.

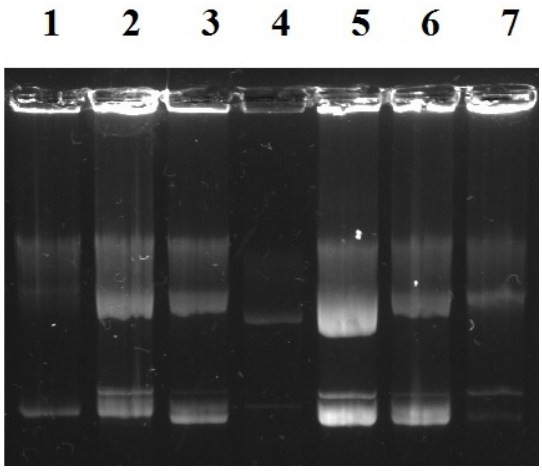

Figure 3. Agarose gel electrophoresis of plasmids isolated from seven *E. coli* DH5α (pAvi-168) transformants (1 to 7).

Plasmids of four *E. coli* DH5α (pAvi-168) transformants were digested with *EcoR*I enzyme and analyzed by agarose gel electrophoreses (Figure 4).

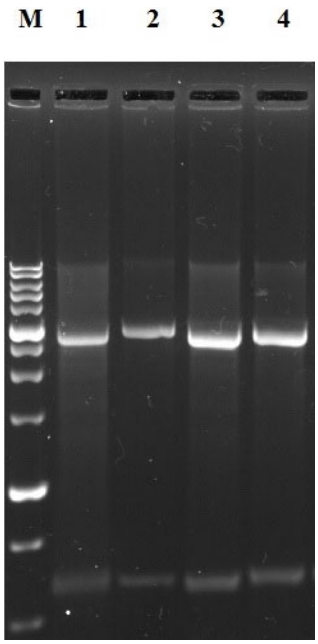

Figure 4. Agarose gel electrophoresis of *EcoR*I digestion of plasmids isolated from four *E. coli* (pAvi-168) transformants. Lane M: 1 kp DNA Ladder GeneON, lanes 1 to 4: Plasmids of four different *E. coli* (pAvi-168) transformants digested by *EcoR*I.

Results obtained from Figure 4 confirmed the existence of only one plasmid in each tested transformant, despite its different forms shown in Figure 3. Figure 4 also presented the *avicelase* gene (lower DNA bands) in all tested transformants, where the *EcoR*I cuts the recombinant plasmid twice at the border of the insert (Figure 1).

3.3. Avicelase Expression:

The iodine method for detecting the avicelase activity was used for testing the two *E. coli* recipient strains, *B. subtillis* 168 the donor strain, and three *E. coli* (pAvi-168) (Figure 5).

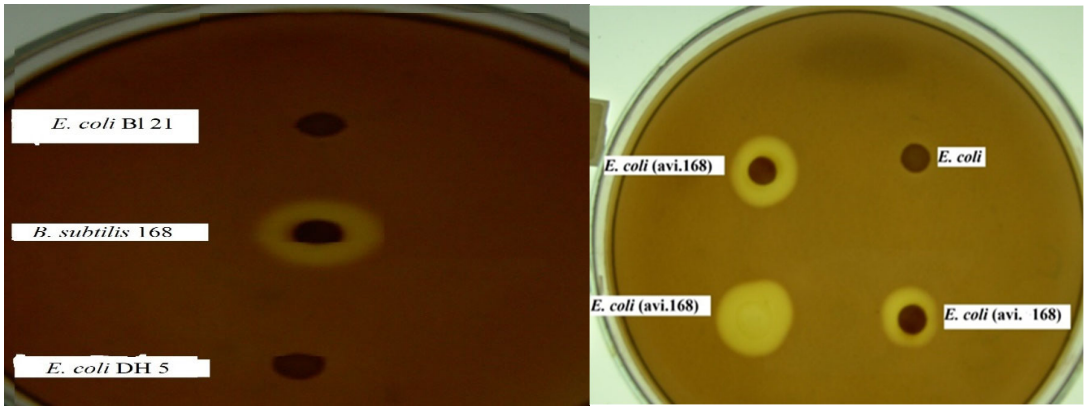

Figure 5. Avicelase activities using iodine detection method.

Figure 5 showed the avicelase activity of the donor strain *B. subtilis* 168 and negative activities of *E. coli* recipient strains (left side of Figure 5). The *E. coli* had acquired avicelase activity after its transformation by the avicelase plasmid (*E. coli* (pAvi-168) (right side of Figure 5). These results indicated the biological activity of the cloned *avicelase* gene.

3.4. Avicelase Activity

The BHM medium supplemented with avicel or rice straw as the sole carbon source was inoculated with an equal inoculum of *E. coli* DH5α (pAvi-168) or *E. coli* Bl 21 (pAvi-168) and then incubated at 37 °C with 120 rpm shaking up to 4 days. Samples from each culture were centrifuged at 12,000 rpm for 3 min and 1 mL of the supernatant was used as aviclease crude enzyme and assayed for its activity as described above. As presented in Figure 6, avicelase activities were found up to the fourth day of incubation with avicel or rice straw in both *E. coli* recombinant strains. The best activity was on day 2 (about 50 IU/L) of *E. coli* DH5α (pAvi-168) with avicel or *E. coli* Bl 21 (pAvi-168) with rice straw. These results agree with [53], who evaluated the effect of different agro-biomass on cellulase production by white rot fungi *P. ostreatus* and *P. chrysosporium* under submerged fermentation. The suitable agro-biomass were wheat straw (6.880 U/mL) and corn stover (6.525 U/mL).

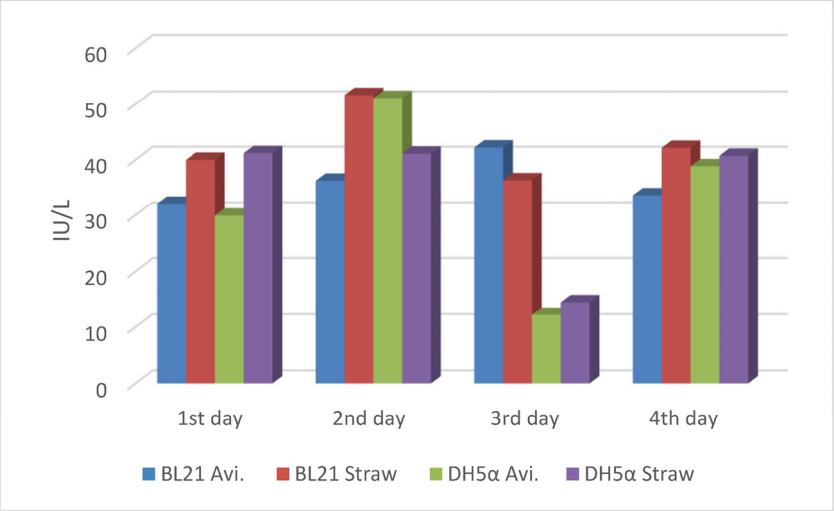

Figure 6. Avicelase activity of two *E. coli avicelase*-recombinant strains grown in BHM medium with avicel or rice straw.

3.5. Optimized Condition for Avicelase Expression

3.5.1. Effect of Rich and Minimal Media, and Shaking on Avicelase Activity

Avicelase activity expression of *E. coli* DH5α (pAvi-168) was studied using an LB medium or BHM medium after incubation at 37 °C with shaking (120 rpm) or static conditions.

Results indicated that the *avicelase* gene was continuously expressed in all treatments without the need for avicel. Table 4 showed different avicelase activities of *E. coli DH5α* (pAvi-168). Shaking was better than the static condition. These results reflect the role of aeration and culture homogeneity on avicelase activity. The rich medium (LB) showed higher avicelase activity than the minimal medium (i.e., BHM); this is more probable because of organic nitrogen and carbon sources in the rich medium. These results agreed with [54], who found that organic nitrogen sources were more suitable for optimizing cellulase production by *Bacillus subtilis* and *Bacillus licheniformis* than inorganic sources.

Results indicated also that when *E. coli DH5α* (pAvi-168) was inoculated in LB with shaking, the free glucose was increased gradually up to the third day of incubation. After the third day, the avicelase activities declined gradually (data not shown).

Table 4. Effect of LB or BHM media with shaking or static incubation on avicelase activity of *E. coli DH5α* (pAvi-168).

Treatment	Avicelase Activity IU/L ± SE *		
	1st Day	2nd Day	3rd Day
LB with shaking	29 ± 0.59	30 ± 0.47	39 ± 0.24
LB static	1 ± 0.18	8 ± 0.47	12 ± 0.47
BHM with shaking	3 ± 0.24	3 ± 0.24	3 ± 0.24
BHM static	3 ± 0.24	1 ± 0.18	1 ± 0.09

* SE standard error.

3.5.2. Effect of Inorganic Nitrogen and pH on Avicelase Activity

The BHM medium was supplemented with different inorganic nitrogen (0.1%) with different pH values, inoculated with *E. coli DH5α* (pAvi-168), and incubated for 3 days at 37 °C with 120 rpm shaking. Then, glucose concentrations were determined.

Results presented in Figure 7 showed that the highest avicelase activities were found when using NH_4NO_3 at pH 6 or 7 and (NH_4) SO_4 at pH 8. The microbial avicelases showed a broad range of optima pHs and temperatures where the optimum pH was 4-6 while the optimum temperature was 40 °C–70 °C [46]. *B. licheniformis* cellulase production [55] was favored in acidic pH, with the most cellulase produced at pH 3.5, where cellulase activity, measured as filter paper activity, (FPase) was 1.07 U/mL. At pH 4 and above, cellulase production was drastically reduced. It has been reported by [56] that acidic pH induces the production of cellulases in thermophilic cellulolytic *Bacillus* sp. The results agreed with [55], who found that *B. licheniformis* had optimal cellulase activity (7.1 U/mL) when ammonium nitrate was the nitrogen source. Additionally, in contrast with the study of [57], who found cellulase activity in another *B. licheniformis* strain, the activity was not enhanced by inorganic nitrogen.

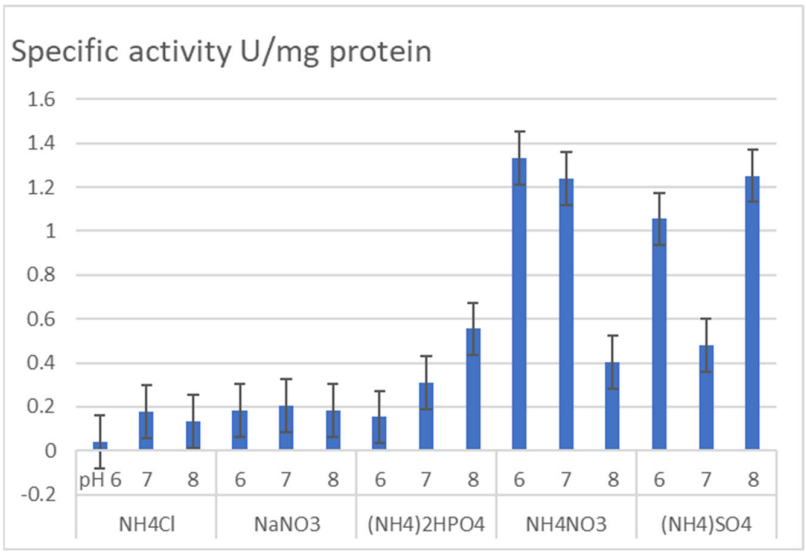

Figure 7. Effect of inorganic nitrogen and pH on avicelase activity of *E. coli DH5α* (pAvi-168) after 3 days incubation at 37 °C, 120 rpm.

3.5.3. Effect of Organic Nitrogen and pH on Avicelase Activity

The BHM *medium* was supplemented with different organic nitrogen (0.1%) with different pH values, inoculated with *E. coli DH5α* (pAvi-168), and incubated for 3 days at 37 °C with 120 rpm shaking. Then, glucose concentrations were determined (Figure 8).

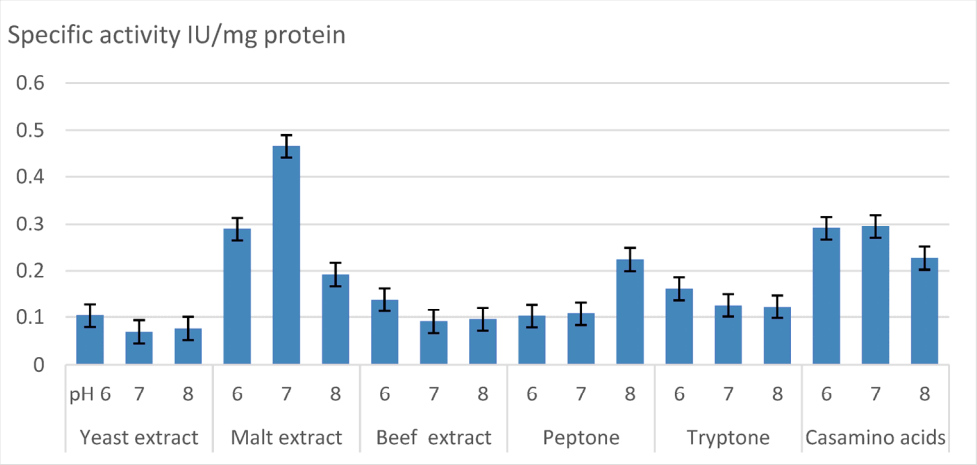

Figure 8. Effect of organic nitrogen and pH on avicelase activity of *E. coli DH5α* (pAvi-168) after 3 days incubation at 37 °C, 120 rpm.

Results revealed that malt extract was the suitable choice of organic nitrogen in avicelase activity, where 0.47 U/mg was reached when used at pH 7.0, followed by casamino acids (about 0.3 U/mg). Double the activity was obtained when peptone was used at pH 8. These findings are in contrast with [55], who found that the optimum levels of FPase (8.7 U/mL) were obtained when peptone was used with *B. licheniformis*, and other organic nitrogen sources did not differ greatly from the control. Karim et al. [57] reported that cellulase production by *B. licheniformis* KIBGE- IB12 increased with increasing peptone and yeast extract concentrations. As mentioned by [58], cellulase activity increased when peptone was used as a nitrogen source by *Bacillus* sp. This situation is more probable due to species-specific preferences in utilizing organic compounds for cellulase production.

In this article, different media and growth conditions, including different carbon and nitrogen sources, as well as pHs and shaking or static conditions, have been studied to identify the optimum conditions for avicelase expression. Results indicated that the *avicelase* gene was continuously expressed without the need for avicel in the medium. The best avicelase activity was on day 2 (about 50 IU/L) of *E. coli* DH5α (pAvi-168) with avicel or *E. coli* Bl 21 (pAvi-168) with rice straw. The fluctuation of avicelase activities within the four days is more probable due to the regulation of its production; that is, more glucose in the medium resulted in less enzyme production and vice versa. The optimum conditions for avicelase activity within *E. coli* DH5α (pAvi-168) showed that shaking, which increases the oxygen level and homogenates the culture contents, and the rich medium (LB) was much better than minimal medium (i.e., BHM). The free glucose was increased gradually up to the third day of incubation. Inorganic nitrogen and pH tests indicated that the highest avicelase activities were found when using NH_4NO_3 at pH 6 or 7 and (NH_4) SO_4 at pH 8. The malt extract was found more suitable than other tested organic nitrogen sources in avicelase activity, where 0.47 U/mg protein was reached with pH 7.0.

3.6. Bioethanol Fermentation

Bioethanol produced from lignocellulosic biomass keeps attracting global attention as alternative energy for oil fuel for a sustainable energy society since the depletion of fossil fuels, rise of global warming, and reduction of natural resources have increased concern [59]. Lignocellulosic biomass arises from agricultural, forestry, and industrial waste. Rice straw is one of the agricultural wastes caused by rice cropping and is a hopeful feedstock for bioethanol production because cellulose and hemicellulose content is more than 50%. Fermenting microorganisms is necessary to achieve efficient ethanol production from such materials [60]. The optimized enzymes were used in the bioconversion of alkali-treated rice straw to fermentable sugar and subsequent ethanol [7].

Avicelase was isolated from the *E. coli* recombinant strain as described above and used to saccharify rice straw. The glucose produced by rice straw was concentrated and supplemented with different nutrients as described above and then inoculated with the *Saccharomyces cerevisiae* F307 strain. After incubated statically at 34 °C for 72 h, ethanol was determined; results are illustrated in Table 5.

Table 5. Bioethanol obtained from rice saccharification.

Parameter	Results ± SE *
Ethanol % (*v*/*v*)	5.26 ± 0.47
Residual sugar	0.09 ± 0.01
Fermentation Efficiency %	86 ± 0.47

* SE standard error.

The ethanol produced was 5.26% (*v*/*v*), the residual sugars were 0.09%, and the fermentation efficiency was 86%.

4. Conclusions

A method for *avicelase* gene amplification from *Bacillus subtilis* was described using the PCR technique, where the annealing temperature at 42 °C was critical. It was cloned with pGEM®-T Easy Vector to produce the recombinant plasmid pAvi-168, which was transferred into each *E. coli* DH5α or *E. coli* Bl 21. The successful transformation was confirmed physically by plasmid extraction and detection after agarose gel electrophoresis and by *avicelase* gene expression.

Effects of nutritional and physicochemical factors were investigated for cellulase production by the *E. coli* transformant, including different carbon and nitrogen sources, as well as pHs and shaking or static conditions. The highest avicelase expression was found on the 2nd day of incubation with avicel, which produced 50 IU/L, followed by using rice straw for 2 days (40 IU/L). Using LB medium with shaking for 3 days produced 39 IU/L. The best inorganic nitrogen source was found to be $(NH_4)2NO_3$ at pH 6 (about 1.4 IU/mg protein, followed by the same compound at pH 7 and with $(NH_4)SO_4$ at pH 8 (more than 1.2 IU/mg protein). The best organic nitrogen was malt extract at pH 7, which produced about 0.45 IU/mg protein.

The recombinant strain was grown in the optimum condition for avicelase production then the enzyme was extracted from culture supernatants and used for saccharification of the rice straw. The glucose syrup obtained was used to grow yeast strains in fermentation conditions for three days to produce bioethanol. The ethanol produced was 5.26% (*v*/*v*), the residual sugars were 0.09%, and the fermentation efficiency was 86%.

The obtained results showed and described the ability to clone one of the cellulolytic genes (i.e., *avicelase*) for the valorization of rice straw for producing renewable energy and bioethanol from cellulolytic wastes. It also identified avicelase production during different nutritional and culture conditions, which is an essential step to develop economically competitive biostrategies, such as biopreparation for efficient cellulosic-waste biodegradation and production of bioethanol and biorefinery products.

Author Contributions: Conceptualization, M.S.A.-S.; methodology, M.S.A.-S., B.E.K., S.S.H. and S.A.H.M.; formal analysis, B.E.K., S.S.H., S.A.H.M. and W.K.H.; data curation B.E.K. and M.S.A.-S.; writing—original draft preparation, M.F., B.E.K., S.S.H. and S.A.H.M.; writing—review and editing, M.S.A.-S. and B.E.K.; supervision M.S.A.-S. and M.F. All authors have read and agreed to the published version of the manuscript.

Funding: This paper is based upon work supported by Science, Technology & Innovation Funding Authority (STDF) under a grant (Pollution call 1) project 41611, Cairo, Egypt.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are included in this article.

Acknowledgments: This paper is based upon work supported by Science, Technology & Innovation Funding Authority (STDF) under a grant (Pollution call 1), Cairo, Egypt. The authors thank Islam Diaa Al Tayyar for proofreading the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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