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Optimization of Growth Medium and Enzyme Assay Conditions for Crude Cellulases Produced by a Novel Thermophilic and Cellulolytic Bacterium, *Anoxybacillus* sp. 527

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Abstract A newly isolated *Anoxybacillus* sp. 527 was found to grow on crystalline cellulose as sole carbon and energy sources. Cellulases secreted by strain 527 were better induced by cellobiose, followed by glucose, lactose, sucrose, and cellulose. Cellulase secretion was enhanced by an optimized medium. Cellulase activity was increased by the addition of Ca^{2+} and NH_4^+ and achieved maximum as 7.0 FPU ml^{-1} at 70 °C and pH6.0. Even at 100 °C, the enzymes were still active, which implies their potential application in large-scale cellulose conversion process.

Keywords Cellulose · Thermophilic · Cellulase · *Anoxybacillus* sp. 527

Introduction

It has been identified that the initial conversion of lignocellulosic feedstocks to sugars is the key bottleneck in the process of biofuel production [1–4]. To achieve this transformation, feedstock pretreatment followed by enzymatic saccharification is favored. Currently, most pretreatment techniques require high temperatures (more than 100 °C) to break down rigid plant cell wall structures. But broadly used cellulases extracted from fungal species have optimal activities at 50 °C. Therefore, pretreated biomass must be cooled down first to this temperature before it can be processed further. This cooling step adds cost and lengthens the treatment time. Moreover, even though cellulases are commercially available, they are still very costly, which severely limits their use and poses a major hurdle for industrial-scale biofuel production from

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biomass [5–7]. Considering all of these factors, thermostable cellulases are actively sought and are one of the critical areas related to cellulase research [8].

Various anaerobic and thermophilic *Clostridium* and *Thermoanaerobium* species have been investigated for their potential use as ethanol producers but have been consistently discovered to suffer from end-product inhibition and membrane damage [9, 10]. Recently, during pH-controlled fermentations, salt accumulation has been observed and determined to be an additional or even overriding barrier to production of ethanol by *Thermoanaerobacterium thermosaccharolyticum*, a thermophilic bacterium that is tolerant to high levels of ethanol [8, 11]. Aerobic cellulose-degrading fungi, for example, *Trichoderma reesei*, has been thoroughly examined over the decades. However, the upper limit of the thermal tolerance of the cellulolytic enzymes from *T. reesei* is 50 °C. On saccharification of cellulose at this temperature, contamination by thermotolerant microorganisms belonging to the genus of *Bacillus* sometimes occurs [12]. Aerobic and thermophilic cellulose-hydrolyzing bacteria have been reported with *Thermobifida fusca* being studied the most [13, 14]. Despite their temperature stability [12], the productivities and properties of their cellulases are not sufficient for effective saccharification of cellulose compared with those produced by *T. reesei*.

Genetic and metabolic engineering have been adopted for creating mutants with exceptional cellulose hydrolyzing capabilities. However, catabolically versatile engineered strains and novel isolates engineered with ethanologenic pathways have subsequently been shown to exhibit limitations in ethanol tolerance, hindering their full potential as economically viable production strains [15]. Also, the overall metabolic energy burden of both ethanol fermentation and cellulase production in an energy-frugal anaerobe is just beginning to be evaluated [16, 17].

Hence, as pinpointed by US Department of Energy in 2008, one of the main challenges for producing cellulosic biofuels is to identify the most efficient thermostable enzymes from fungi or bacteria from the natural environment [18]. That is to say that much emphasis is still placed on the screening of new strains with high specific cellulase activities and better physico-chemical properties than those currently obtained. The objective of this study was to gain better understanding of a thermophilic, cellulolytic, and facultatively aerobic *Anoxybacillus* sp. 527 isolated from swine waste. Optimal growth medium for cellulase secretion, optimal conditions for cellulase activities, and sugar induction effects are reported here.

Materials and Methods

Chemicals

Avicel cellulose (PH-105) was kindly provided by FMC BioPolymer (Philadelphia, PA, USA). Yeast extract, tryptone, and peptone were purchased from Difco laboratories (Sparks, MD, USA). Glucose, cellobiose, lactose, and sucrose were bought from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of the highest grade possible from Fisher Scientific (Pittsburgh, PA, USA) if not noted specifically.

Isolation and Identification

Several years ago, a pilot plant of 3.8 m^3 was built to treat swine waste from a swine-finishing building at Southern Illinois University Carbondale using aerobic and thermophilic (57 °C) degradation process. Sample from this reactor was lyophilized and used as the original source

for isolating cellulose-degrading bacteria. The procedure for isolation and identification of an *Anoxybacillus* sp. was basically the same as what was reported for identification of a cellulose-degrading *Brevibacillus* sp. JXL [19]. Briefly, 0.5 g freeze-dried bacterial source sample was added to 50 ml 0.85% sterile NaCl solution. The mixture was homogenized on a shaker at 150 rpm for 1 h at 57 °C. Ten milliliter of the mixture was inoculated into 90 ml medium containing (per liter): $NaHCO_3$ (2.06 g), NH_4Cl (0.68 g), K_2HPO4 (0.296 g), KH_2PO4 (0.18 g), $(NH_4)_2SO_4$ (0.15 g), $MgSO_4{\cdot}7H_2O$ (0.12 g), $CaCl_2{\cdot}2H_2O$ (61.0 mg), $FeSO_4{\cdot}7H_2O$ (21.0 mg), NaCl (10.0 mg), $MnSO_4{\cdot}H_2O$ (5.0 mg), $CoCl_2{\cdot}H_2O$ (1.0 mg), $ZnSO_4{\cdot}7H_2O$ (1.0 mg), $CuSO_4{\cdot}5H_2O$ (0.1 mg), H_3BO_3 (0.1 mg), $KAl(SO_4)_2{\cdot}12H_2O$ (0.1 mg), $Na_2MoO_4{\cdot}2H_2O$ (0.1 mg), L-cysteine–$HCl{\cdot}H_2O$ (0.25 g), and Wolfe's vitamin solution (American Type Culture Collection, 10 ml) [20]. After the enrichment culture supplemented with Avicel was incubated at 57 °C for 48 h, 100 μl of samples were spread on cellulose agar plates made with the same medium as identified above but with the addition of agar (15 g l^{-1}). The plates were then put in the same incubator. After 72 h, around 40 colonies appeared on the plate. One colony was chosen for further purification based on the size of a clear zone visible after Congo red staining. To identify this isolate, the 16 S rDNA was amplified by polymerase chain reaction using the same program as demonstrated in [19].

Nucleotide Sequence

The partial 16S rDNA sequence (1,420 bp) of the isolate has been submitted to Genbank. The accession number is FJ744749.

Isolate Maintenance

Due to the complexity of the enrichment medium described above, a much simpler medium designated as medium I was initially used for studying this isolate. Medium I contained (per liter): 1.0 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.5 g *l*-asparagine, 0.5 g KCl, 0.5 g yeast extract, 0.2 g $MgSO_4$, and 0.1 g $CaCl_2$ [20]. This isolate has been maintained on cellulose agar plate and in frozen stock (15% glycerol) at −80 °C. Upon starting a new experiment, 200 μl frozen stock was added to 50 ml Luria–Bertani medium (per liter): 10 g tryptone, 5 g yeast extract, and 10 g NaCl. After overnight growth at 57 °C on a rotary shaker set at 150 rpm, the log-phase culture served as an inoculum for different experiments.

Medium Optimization: Effect of Different Nitrogen Sources and Various Other Chemicals on Cellulolytic Activity

In order to develop a more suitable medium for cellulase secretion, different chemicals were screened to improve the medium I. Nitrogen sources such as sodium nitrate (1 g l^{-1}), ammonium sulfate (0.5 g l^{-1}), yeast extract (1 g l^{-1}), tryptone (1 g l^{-1}), and peptone (1 g l^{-1}) were evaluated. Cellobiose at 10 g l^{-1} was used as the carbon source. Cultures (100 ml in 250-ml flask) were incubated at 57 °C on a rotary shaker set at 150 rpm. This incubation condition was used throughout the rest of studies reported here. After cell growth for 72 h, supernatant, also referred to as crude enzymes after centrifugation at 18,000×*g* for 10 min from these cultures, were examined for filter paper unit (FPU) based on Ghose's procedure [21]. In short, 0.5 ml of crude enzyme solution was added to 1.0 ml citrate buffer (50 mM, pH4.8) in a test tube with a filter paper strip (Whatman no.1, 1×6 cm). After incubation at 50 °C for 1 h, the concentration of reducing sugars released from filter paper degradation

was determined by dinitrosalicylic acid reagent using glucose as a standard. One unit of FPU was defined as 1 μmol glucose equivalents formed per minute.

Once the optimal nitrogen source was determined, it was added to final medium II. Effects of vitamins and surfactants were also tested on cellulase secretion. These chemicals included (per liter): thiamine·HCl (0.05 mg) and D-biotin (0.02 mg). Two surfactants including Triton-X 100 and Tween 80 were evaluated at doses of 1, 1.5, or 3 g l^{-1}. Individual chemical was added to medium I with the presence of cellulose (10 g l^{-1}). After 24 h, supernatant sample from each flask was tested in terms of FPU. Again, chemicals having positive effects on cellulase activity were incorporated into the final medium II.

FPU Assay Condition Optimization: Effect of Ca^{2+}, Temperature, pH, and Other Chemicals on Cellulase Activity

To validate calcium effect on cellulase activities, suspension samples obtained from strain 527 grown on cellobiose at 7.5 g l^{-1} for 24 h were adopted. Ca^{2+} concentrations from 0 to 10 mM were added to the Tris–HCl buffer (pH6.0) used in the activity assay at 50 °C. The best dose of Ca^{2+} was then applied in subsequent FPU analysis.

To determine the optimal pH and temperature for the crude cellulases produced by strain 527, medium I was supplemented with glucose and cellulose each at 10 g l^{-1} and incubated for 24 h. The suspension sample after allowing the flasks to sit at bench for 15 min was subjected to FPU analysis. First, the FPU assay was conducted at pH6.0 with temperatures ranging from 50 to 100 °C. The optimal temperature was then used to inquire the pH effect from 4 to 8. The following buffers were used: pH4 to 5 with 50 mM citric acid–NaOH and pH6 to 8 with 50 mM Tris–HCl. The best temperature and pH were employed for later FPU assays.

Besides the effects exerted from Ca^{2+}, pH, and temperature on cellulase activities, we also investigated the effects of other chemicals at different doses based on what have been reported by other researchers [22–24]. These chemicals were: $CoCl_2$ (1 and 5.5 mM), $MnCl_2$ (1 and 5.5 mM), NH_4Cl (5.5 mM), $MgCl_2$ (5.5 mM), NaCl (5.5, 7, 10 mM), NaN_3 (0.5, 1.0, 1.5, 2.0 g l^{-1}), and dithiothreitol (DTT; 5, 10, 15, 20 mM). Chemicals that had the positive effects were incorporated into the final buffer recipe for FPU assay.

Cellulase Induction by Different Carbohydrates

Different fermentable sugars have been shown to either induce or inhibit cellulase production depending on individual species. To decipher the sugar effect for strain 527, we compared the cellulase activities of supernatant samples attained from cultures grown on either glucose, cellobiose, lactose, sucrose, or cellulose in medium I. Doses from 2.5 to 12.5 g l^{-1} for each substrate were assessed. After 48 h growth, cultures were harvested and centrifuged. The supernatant samples were used for FPU determination. All determinations were repeated at least twice. Representative results are reported below.

Medium II Development and Testing

Based on the effects of various nitrogen sources, surfactants, and vitamins, a final medium II was developed. Medium II (pH5 and 6) was used to test activities of crude enzyme solutions from cultures grown on mixed substrates including glucose (2.5 g l^{-1}), lactose (5 g l^{-1}), and Avicel (5 g l^{-1}). The suspension samples were taken at 12, 24, 30, and 48 h and centrifuged at 18,000×*g* for 10 min. Supernatant from each sample was diluted for FPU assay.

Results and Discussion

Isolation and Identification

The partial 16S rDNA sequence was obtained with a length of 1,420 bps. The phylogenic tree illustrating the relationship of this isolate with other *Anoxybacillus* species and a selected group of cellulose-degrading bacteria is depicted in Fig. 1. The taxonomical assignment of this bacterium is 100% *Anoxybacillus* genus in the *Bacillaceae* 1 subfamily within the *Bacilli* class of the *Firmicutes* phylum based on the classification provided by Ribosomal Database Project Release 10. BLAST search of the sequenced 16S rDNA indicates that this isolate is 99% identical to *Anoxybacillus beppuensis* strain TSSC-1 (EU710556) isolated from clay soil sample in the vicinity of a hot water reservoir in Tulasi Shyam, India; 99% identical to *A. beppuensis* (AB243446.1), a heat-stable protease producer isolated from a hot spring in Japan; and 98% identical to *Geobacillus* sp. K6 (AM749790.1) isolated from geothermal soils in the Taupo Volcanic Zone of New Zealand [25]. Thus, this new isolate is highly related to other thermophilic bacteria. But, to the best of our knowledge, there are no reports about the capability of celluose degradation and secretion of cellulase enzymes by *A. beppuensis* and *Geobacillus*. Among the genus of *Anoxybacillus*, only the species of *Anoxybacillus flavithermus* has shown cellulose and xylan-degrading abilities [26, 27].

Medium Optimization

Various nitrogen sources were proved to have different influences on carbohydrate degradation [28–35]. In this study, the presence of yeast extract, tryptone, or peptone in the culture medium enhanced cellulase activities more compared with inorganic nitrogen sources as shown in Table 1. This enhancement may be due to other nutrients and growth stimulators in the organic nitrogen source besides nitrogen [36]. When peptone was the nitrogen source, the enzymatic activity was the highest. The lower cellulase activity attained with inorganic nitrogen suggests decreased utilization of it by strain 527. This is in agreement with the finding from anaerobic bacterium *Clostridium papyrosolvens* CFR-703 [37].

In this study, the two non-ionic surfactants, Triton X-100 and Tween 80, both increased the cellulase activities with Triton X-100 showing higher effect at 10 g l^{-1} (Table 2). Triton X-100

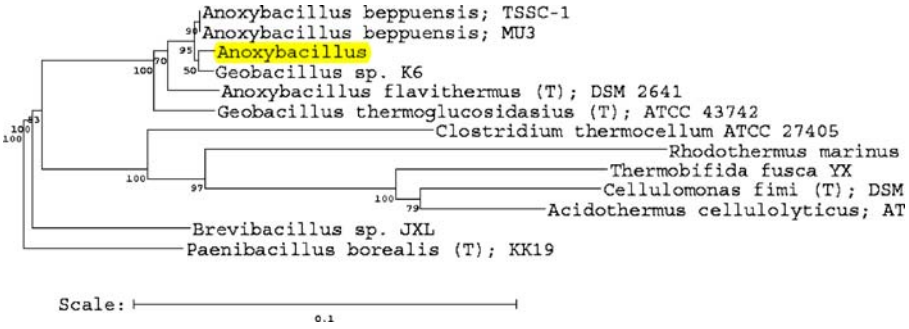

Fig. 1 Phylogenetic tree based on 16S rDNA sequences of *Anoxybacillus* sp. 527, its closest relatives, and a selected group of cellulose-degrading microbes (sequences from the Ribosomal Database Project Release 10). The tree was constructed using Weighbor Tree Method (a distance-corrected modification of Jukes Cantor). *Numbers at the nodes* indicate bootstrap values based on 100 replicates, while the *scale* indicates number of changes per nucleotide position

Table 1 Comparison among different nitrogen sources in the medium on crude cellulase activities.

Nitrogen source	Relative FPU[a]
$NaNO_3$	100
$(NH_4)_2SO_4$	107
Yeast extract	113
Tryptone	126
Peptone	173

Cellobiose at 10 g l^{-1} served as the carbon source

[a] When NaNO3 was used as the nitrogen source, an enzymatic activity as 0.129 U ml^{-1} tested at 50 °C and pH4.8 was detected for the crude enzyme solution and set as 100. This was the basis for calculating relative FPU for samples taken from cultures with other nitrogen sources

was selected into the final medium II. The increased enzymatic activity by non-ionic surfactants is attributed to the unmasking of SH groups in the microbial enzymes and subsequently augmenting the interactions between enzymes and potential substrate [38]. However, effects from surfactants have been controversial. On one hand, Tween 80 elevated cellulase production by *Nectria catalinensis*, while Tween 20 and Triton X-100 inhibited growth and cellulolytic enzyme production by the same species [39]. On the other hand, in terms of impacts on cellulose enzymatic hydrolysis, the reported results are all positive on reducing the enzyme activity loss caused by nonproductive adsorption [40, 41]. Vitamins can stimulate cellulase secretion to some extent. With regard to strain 527, the addition of D-biotin and thiamine increased cellulase activities. For 12 strains of *Cellulomonas* tested in another study, filter paper breakage was enhanced by the addition of D-biotin and thiamine [42]. Hence, these two vitamins were also included into the final medium II.

The final medium II was formed by adding the following components to the medium I identified above. These extra components are (per liter): 1 g peptone, 1 g Triton X-100, 0.05 g thiamine, and 0.02 mg D-biotin.

FPU Assay Condition Optimization

As shown in Fig. 2, the activity of crude cellulases increased with increasing concentration of Ca^{2+} until 5.5 mM, then sharply decreased. Therefore, 5.5 mM was the optimal

Table 2 Effects of surfactants and vitamins added to the medium on enzyme activities.

Additive	Relative FPU[a]
Control[b]	100
Thiamine	138
D-Biotin	148
Triton-X100	152
Tween 80	128

Avicel at 10 g l^{-1} served as the substrate

[a] When strain 527 was grown on Avicel (10 g l^{-1}), an enzymatic activity as 0.019 U ml^{-1} was detected for the crude enzyme solution and set as 100. This served as the basis for calculating relative FPU for samples taken from cultures with other supplements

[b] The control was the medium I identified in this study without any additives

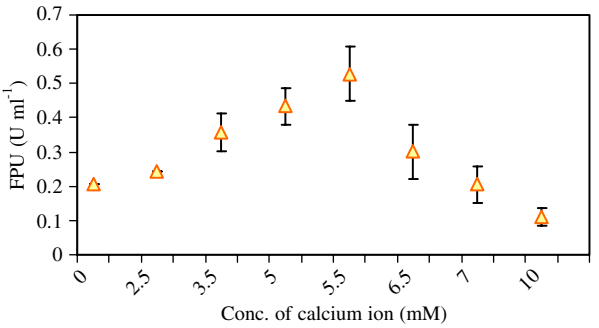

Fig. 2 Effect of Ca^{2+} in buffer on crude enzyme activities. Filter paper unit (FPU) assay was conducted at 50 °C in Tris–HCl buffer (pH4.8)

concentration for Ca^{2+}. Effect of Ca^{2+} has also been reported by other researchers. For example: (1) Ca^{2+} can enhance carboxymethylcellulase (CMCase) activity by 20% compared with that of the control [43]; (2) in the presence of Ca^{2+} (5 mM), the activity of an endoglucanase from *Clostridium thermocellum* is stimulated [44]; and (3) with 5 mM Ca^{2+}, cellulose hydrolysis by enzymes from *C. thermocellum* proceeds to completion [22].

The cellulase enzymes produced by strain 527 demonstrated good activities between 50 and 70 °C with the maximum activity at 70 °C (Fig. 3), which is the same as that of xylanase and close to cellulases from *A. flavithermus* [26, 27]. Notably, although the activities were abruptly reduced at 80 °C, 25% of the maximum cellulolytic potential was still retained even at 100 °C, rendering the cellulases very promising for future protein engineering to further improve their thermotolerance for cellulose degradation at high temperatures. The crude enzymes were active at a broad range of pH (5–7) with optimal pH as 6.0 which is close to the optimum pH values of most *Bacillus* cellulases (Fig. 4) [45].

We analyzed the effects of minerals and a reducing agent-DTT in the buffer for FPU assay. As indicated in Table 3, different chemicals had different efficacy on cellulase activities. While Co and Mn completely inhibited the enzyme activities, the existence of NH_4^+ and Mg^{2+} enhanced enzymes' cellulolytic activities followed by Na^+. These cations might be probably involved in the protection of the enzymes or strengthening of the active sites, thereby maintaining the conformation of the enzymes in active states. According to Simeon [43], Mg^{2+} enhances CMCase by 18% compared with that of the control. In addition, an endoglucanase from *C. thermocellum* is stimulated by 100% in the presence of Mg^{2+} [24]. NaCl and NH_4Cl are also found to be stimulative toward crude cellulases activities. The activity of an endoglucanase from *Bacillus agaradhaerens* JAM-KU023 is increased around 4-fold and 2- to 4-fold by the addition of 0.2–2.0 M NaCl and NH_4Cl, respectively [23].

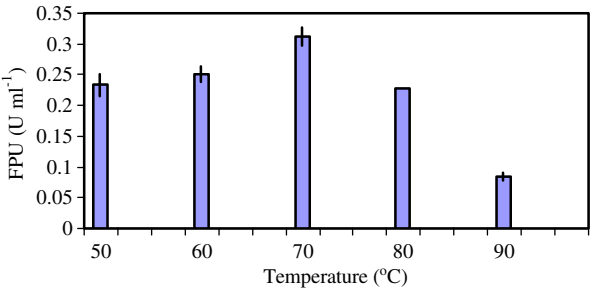

Fig. 3 Effect of temperature on crude cellulase activities. Filter paper unit (FPU) assay was conducted at pH6.0 with temperatures ranging from 50 to 100 °C

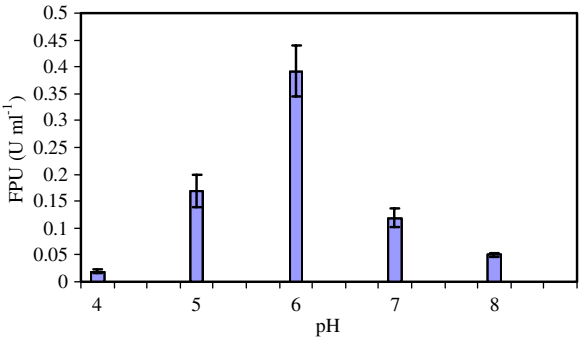

Fig. 4 Effect of pH on crude cellulase activities. Filter paper unit (FPU) assay was conducted at 70 °C with pH ranging from 4 to 8

Sulfhydryl-reducing compounds like DTT was shown to have significant positive effect on hydrolysis of Avicel [22]. DTT is believed to be necessary for reducing the oxidized active site of a cystein residue for α-glucosidase from *Thermotoga maritima* [46]. However, regarding cellulases secreted by strain 527, DTT inhibited cellulase activities. Since there was no pre-incubation of the enzymes with DTT, inactivation must have occurred rapidly, suggesting the presence of essential thiol groups in the enzymes. This is in agreement with findings from *B. agaradhaerens* [23].

Cellulase Induction

As illustrated by Table 4, different carbohydrates can induce cellulase secretion to various extents. Cellobiose, glucose, lactose, and sucrose were more effective inducers compared with cellulose. Cellobiose at 7.5 g l^{-1} produced the highest cellulase activities. Similar results were demonstrated for *T. reesei* [47] and a *Bacillus* bacterium [48]. Growth of this *Bacillus* sp. in medium containing cellobiose or glucose resulted in the greatest

Table 3 FPU assay results affected by the presence of various minerals and a reducing agent DTT in the buffer.

Additive	Relative FPU[a]
Control[b]	100
$CoCl_2$ (1 mM)	0
MnC2 (1 mM)	0
NH_4Cl (5.5 mM)	148
$MgCl_2$ (5.5 mM)	138
NaCl (5.5 mM)	116
NaN_3 (0.15%)	111
DTT (10 mM)	69

[a] Using crude enzyme solution from strain 527 culture supplemented with glucose 2.5 g l^{-1}, lactose (5 g l^{-1}), and Avicel (5 g l^{-1}), a FPU as 0.95 U ml^{-1} was determined under the control condition and set as 100. Relative FPU was obtained by using the same enzyme solution but different buffer conditions with different additives. Different doses of the chemicals listed above have been tested with only the highest values reported here

[b] The control was the Tris–HCl buffer (pH6.0) with Ca^{2+} (5.5 mM) for FPU analysis

Table 4 Induction effects from different carbohydrates on crude cellulase activities.

[a] FPU assays were conducted at 70 °C and pH6.0. Different doses of different carbohydrates have been tested with only the highest values reported here

Substrate (g l^{-1})	FPU (U ml^{-1})[a]
Glucose (12.5)	0.43
Cellobiose (7.5)	0.55
Lactose (10)	0.4
Sucrose (12.5)	0.14
Cellulose (10)	0.017

production of cellulolytic activity. Extracellular protein produced by this *Bacillus* isolate showed roughly one fifth the cellulolytic activity displayed by *T. reesei* C30 on noncrystalline cellulosic substrates [48]. In another study, cosubstrates of Solka Floc and lactose-stimulated cellulase secretion more effectively than Solka Floc alone, and the effect was increased with increasing concentrations of lactose from 0.5% to 1.0% [49].

When both glucose and lactose were fed to medium II with cellulose, much higher cellulase activities were seen compared to those from single substrate (Fig. 5). During the first 30 h, medium with pH6 produced higher enzyme activities compared to those from pH 5. But at 48 h, the activity of crude enzymes from culture with pH5 reached 7.0 U ml^{-1}, approximately 3-fold increase compared with that from culture pH6. For both cultures, the cellulase activities increased with time. Therefore, medium II at pH5 was better suited for cellulase secretion for strain 527. The investigation of the mechanism responsible for higher activities produced by glucose and lactose together is ongoing.

Conclusions

Besides *A. flavithermus*, strain 527 is the only known cellulose degrader in the genus of *Anoxybacillus.* Strain 527 is facultative and thermophilic. It could utilize crystalline cellulose as the only carbon and energy source. Grown on different fermentable sugars and cellulose, strain 527 was observed to secrete cellulases into the medium. Crude cellulases had maximal activity at 70 °C and pH6.0. The medium for enhancing enzyme secretion and the buffer for testing FPU have been optimized for this isolate.

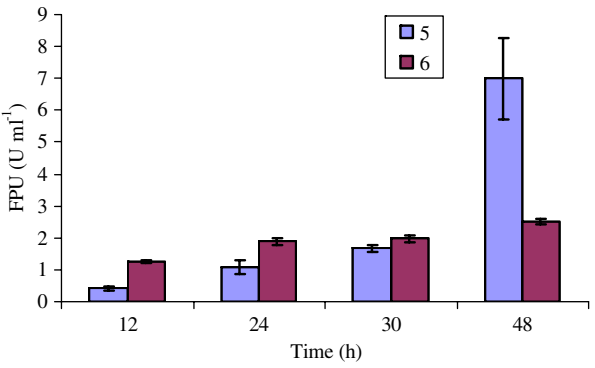

Fig. 5 Adopting the optimized medium and buffer conditions for filter paper unit (FPU) assay, crude enzyme activities were observed to increase with time for strain 527 cultures with an initial pH5 and 6

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