Citrobacter freundii as a test platform for recombinant cellulose degradation systems

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Running Headline

Citrobacter freundii for biomass degradation

Significance and impact of the study

Biofuels have been shown to be the best sustainable and alternative source of fuel to replace fossil fuels. Of the different types of feedstocks used for producing biofuels, lignocellulosic biomass is the most abundant. Converting this biomass to useful products has been met with little success. Different approaches are being used and microbial platforms are the most promising and sustainable ways. This study shows that *Citrobacter freundii* is a better test platform for testing various combinations of cellulases for the development of microbial systems for biomass conversion.

Abstract

Cellulosic biomass represents a huge reservoir of renewable carbon, but converting it to useful products is challenging. Attempts to transfer cellulose degradation capability to industrially useful microorganisms have met with limited success, possibly due to poorly understood synergy between multiple cellulases. This is best studied by co-expression of many combinations of cellulases and associated proteins. Here we describe the development of a test platform based on *Citrobacter freundii*, a cellobiose-assimilating organism closely related to *Escherichia coli*. Standard *E. coli* cloning vectors worked well in *C. freundii*. Expression of cellulases CenA and Cex of *Cellulomonas fimi* in *C. freundii* gave recombinant strains which were able to grow at the expense of cellulosic filter paper or microcrystalline cellulose (Avicel) in a mineral medium supplemented with a small amount of yeast extract. Periodic physical agitation of the cultures was highly beneficial for growth at the expense of filter paper. This provides a test platform for the expression of combinations of genes

encoding biomass degrading enzymes to develop effective genetic cassettes for degradation of different biomass streams.

Keywords

Biofuels, Biodegradation, Gene expression, Recombinant protein, Plasmids

Introduction

It is widely considered that society should move away from dependence on fossil carbon for fuels and bulk chemical feedstocks, and move towards use of renewable materials (Nuffield Council on Bioethics 2011; Creutzig *et al*. 2015; Fulton *et al*. 2015). Plant and algal biomass is the only feasible renewable source available in sufficient quantities to replace a significant fraction of our current use of fossil carbon (Heaton *et al*. 2008; French 2009). However, difficulties in the conversion of biomass to useful chemicals make such processes generally uneconomic at present.

Degradation of cellulose by chemical or enzymic means is relatively slow and difficult due to its insoluble and partially crystalline nature. In the paradigmatic enzymic process, based on studies of organisms such as *Trichoderma reesei*, cellulose chains are first nicked by endoglucanases to expose a free reducing end and non-reducing end. These are then attacked by processive exoglucanases (cellobiohydrolases), releasing cellobiose, which is then hydrolysed by β-glucosidases to yield glucose (Lynd *et al*. 2002). Ideally, a commercial bioconversion process would be accomplished by a single organism, which could produce cellulose-degrading enzymes, assimilate the resulting sugars, and convert them to useful products. A process of this type is referred to as 'consolidated bioprocessing' (Lynd *et al*. 2005). However, most native cellulose-degrading organisms do not produce useful products in high yield, and attempts to transfer cellulose-degrading ability to industrially useful organisms have met with very limited success (French 2009); in particular, growth of such recombinant organisms at the expense of crystalline cellulose does not seem to have been reported, though some strains have been reported to grow with amorphous substrates such as CMC (carboxymethyl cellulose) and PASC (phosphoric acid swollen cellulose) (French *et al*. 2013).

Examination of the genomes of effective cellulose-degrading bacteria reveals that they possess a battery of biomass degrading enzymes, generally including multiple putative endoglucanases, exoglucanases and β-glucosidases. For example, the Gram positive facultative anaerobe *Cellulomonas fimi* has at least four characterized endoglucanases (CenA, CenB, CenC, and CenD), three exoglucanases (Cex, CbhA, and CbhB), and two β-glucosidases. It is suggested, based on the genome sequence, that 11 enzymes were involved in cellulose degradation in *C. fimi* (Christopherson *et al*. 2013), with many others involved in degradation of other polysaccharides; our own examination of the genome sequence (S. Kane and C. French, in preparation) indicates approximately seventy genes having putative roles in liberation of sugars from biomass. This suggests that intra-class and inter-class synergy play a poorly understood role in biomass degradation.

One way to address this question is to use the modular assembly tools of synthetic biology, such as the BioBrick RFC10 assembly system (Knight 2003), to assemble many different combinations of genes encoding biomass degradation enzymes and test these *in vivo* for activity against different biomass substrates. As a first step, it is necessary to develop a test platform which can indicate effective cellulose degradation by growth. Here we describe such a test platform based on *Citrobacter freundii*, a close relative of *Escherichia coli* which possesses the native ability to assimilate cellobiose.

Results and discussion

Development of Citrobacter freundii as a host system.

In order to test synergistic effects in mixtures of cellulases and associated proteins expressed in heterologous systems, we required a test platform for enzyme expression. Due to the wide variety of vectors and techniques available, *E. coli* would be the obvious choice; however, most *E. coli* strains are not able to assimilate cellobiose. Either generation of a special host strain (Kachroo *et al*. 2007), or inclusion of β-glucosidases in mixtures, would thus be required. As an alternative, we investigated close relatives of *E. coli* which have the native ability to assimilate cellobiose. The majority of close relatives of *E. coli*, including *Klebsiella oxytoca*, which has previously been used in such experiments (Ingram *et al*. 1999; Zhou and Ingram 2001) are classified as ACDP (Advisory Committee on Dangerous Pathogens) level 2 in the United Kingdom; other cellulose-assimilating relatives such as *Pantoea* spp. are plant pathogens in which addition of cellulases might alter pathogenicity. We chose to test *C. freundii*, a close relative of *E. coli*, which, though capable of causing opportunistic infections under certain conditions, is not normally considered pathogenic and it is classified as ACDP level 1. *C. freundii* has previously been little used as an expression host (Jiang *et al*. 2010) but has been considered for industrial scale use in manufacture of propane-1,3-diol (Kaur *et al*. 2012).

Two strains were chosen for preliminary testing: *C. freundii* SBS197, from the local teaching laboratory strain collection, and *C freundii* NCIMB11490 (ATCC 8090), the type strain. The source from which the SBB197 strain was isolated is not known. Since the provenance of this strain could not be established, the 16S rRNA gene sequence was amplified and sequenced to confirm its identity. A near full-length

sequence of 1447 nucleotides was amplified using primers fD1 and rD1 (Weisburg *et al*. 1991) and sequenced from both ends. The identity was determined by submitting the sequence to the ribosomal database project (Cole *et al*. 2014) and performing a, NCBI GenBank Nucleotide BLAST. The assembled sequence has been deposited in the GenBank with accession number KX774629. This was found to be identical to that of known *C. freundii* strains with 99% to 100% identity. The whole genome sequences of both strains were found to be substantially similar, though with significant differences. A higher quality genome sequence for the type strain was later submitted to GenBank (accession ANAV00000000) (Kumar *et al*. 2013).

In contrast to our expectations, neither strain possessed a Type II secretion system, though both possessed a Type I secretion system, apparently associated with a large cell surface protein bearing a putative Type I recognition sequence in its C-terminal region (WP_003839819). Strain NCIMB11490 also appeared to possess a second Type I secretion system, possibly associated with a heme peroxidase, whereas SBS197 appeared to possess components of Type III and Type VI secretion systems. Both strains possessed apparent cellobiose phosphotransferase systems and multiple 6-phospho-β-glucosidases, consistent with their demonstrated ability to assimilate cellobiose. In addition, each strain possessed at least one putative periplasmic β-glucosidase.

To assess the range of *E. coli* replicons which could be used in *C. freundii*, other plasmids (Table 1) from the Registry of Standard Biological Parts were introduced into *C. freundii* NCIMB11490. *C. freundii* NCIMB11490 was found to be highly transformable, with transformation efficiencies comparable to those seen in *E. coli*

JM109 using the same method. However, *C. freundii* SBS197 showed much lower transformation efficiencies, with typically fewer than five colonies recovered in each transformation experiment, even with excess (>100 ng) supercoiled plasmid DNA. Expression of RFP led to visibly red colonies, as in *E. coli* (Additional comments in supplementary information).

Preparing and testing cellulase cassettes

As a source for well characterized cellulases, we chose to work with *Cellulomonas fimi*. While the genome sequence was not available at that time, several well studied cellulases had been cloned, and one combination in particular, CenA (endoglucanase) plus Cex (exoglucanase), had been shown to liberate reducing sugars from wood chips when expressed in a heterologous host, *Saccharomyces cerevisiae* (Wong *et al*. 1988). We therefore prepared a genetic cassette of *cenA-cex* in BioBrick form under the control of *lac* and *spac* promoters for expression in *C. freundii* and *E. coli*.

Extracellular CenA and Cex activity were present in the test strains but absent in control strains bearing only the same plasmid with *lac* promoter (Supplementary Fig. 1a). Quantitative CenA and Cex assays showed that a significant amount of enzyme activity was present in the culture supernatant of *C. freundii* whereas in a similar experiment with *E. coli* JM109, enzyme expression was mainly intracellular (Fig. 1 and Supplementary Fig. 1b). 'Leakage' of both CenA and Cex from *E. coli* (Guo *et al*. 1988) and *Caulobacter crescentus* (Bingle *et al*. 1993) has previously been reported; the mechanism is not clear, but a number of possibilities have been proposed (Ni and Chen 2009). We are currently investigating the possibility of using the native Type I secretion system of *C. freundii* for cellulase secretion.

Testing growth on cellulosic substrates

To determine whether expression of these cellulases led to growth at the expense of cellulosic substrates, cultures were grown in minimal medium (MM1) supplemented with filter paper, or Avicel (powdered microcrystalline cellulose) as main carbon source. Recombinant *E. coli* JM109 expressing the cellulases were not able to utilize either filter paper or Avicel as a carbon source. Although there was some breakdown of filter paper after 4-8d, growth did not appear to be enhanced (Supplementary Fig. 3). This inability could be due to the reason that *E. coli* does not produce β-glucosidase which is required to break down cellobiose that is released after endoglucanases (eg. CenA) and exoglucanases (eg. Cex) act on cellulose. *C. freundii* on the other hand was able to utilize filter paper and Avicel as a main carbon. Cultures expressing cellulases showed enhanced growth in the presence of cellulosic substrates, compared to controls lacking cellulases or with no additional substrate supplied (Fig. 2).

Initial growth experiments with filter paper were disappointing. However, through an accident, it was noted that violent agitation of cultures led to rapid destruction of filter paper in cellulase-expressing strains, but not in control strains. To test the hypothesis that violent physical treatment in combination with cellulase expression was required for disruption of cellulose fibres, test cultures were agitated on a vortex mixer for 60 seconds once every 24 hours. This led to rapid, reproducible destruction of filter paper in cultures expressing cellulases after 24 to 48 hours growth, but not in cultures which did not express cellulases, in which only a slight rounding of the corners of filter paper squares was observed, even after prolonged incubation (7 d). In cultures that

were vortexed for one minute every 24 hours, filter paper was destroyed within 48 hours (Supplementary Fig. 2) and growth was enhanced when assessed based on the area under the growth curves (Fig. 3a). This agitation was found to have little effect on cultures provided with Avicel which is a powdered form of cellulose. Agitation has previously been reported to have a strong effect on CenA activity in processing of cotton fibres for textiles (Azevedo *et al*. 2000). Our results suggest that periodic violent agitation may generally be beneficial in biomass degradation experiments.

It was reproducibly observed that cultures expressing cellulases showed greatly enhanced colony counts compared to controls which did not express cellulases, or which were not provided with such substrates. This was not found for when *E. coli* was used as an expression host, thus we conclude that *C. freundii* appears to be a suitable test platform for testing cellulase combinations.

Materials and Methods

Organisms and growth conditions

Genetic manipulation procedures were performed in *Escherichia coli* JM109. *Citrobacter freundii* strain SBS197 was obtained from the School of Biological Sciences teaching laboratory culture collection, University of Edinburgh. *C. freundi* NCIMB11490 (ATCC8090), the type strain, was obtained from NCIMB, Aberdeen, UK. *Bacillus subtilis* 168 was obtained from Dr. Garry Blakely, University of Edinburgh. *Cellulomonas fimi* ATCC484 (type strain) was obtained from DSMZ, Germany. All organisms were grown at 37˚C. Liquid cultures were incubated on a rotary shaker at 180 rpm. Organisms were maintained on Luria Agar (LA) or Nutrient

Agar (NA) with appropriate antibiotics as indicated below. Chloramphenicol (Duchefa Biochemie, The Netherlands) for selection recombinant *C. freundii* was added to media at a final concentration of 15 μg ml^{-1} whereas for *E. coli*, it was 40 μg ml^{-1}. The 40 μg ml^{-1} was found to impair the growth of *C. freundii* and by experimentation, 15 μg ml^{-1} was found to be optimal. Carbenicillin (Melford Laboratories, UK), Kanamycin (AppliChem GmbH, Germany), Tetracycline (Duchefa Biochemie, The Netherlands), and Ampicillin (Melford Laboratories, UK) were added media at a final concentrations of 80, 50, 5 and 100 μg ml^{-1} respectively.

Cellulose degradation experiments were initially conducted in Minimal Medium 1 (MM1) consisting of 2 g l^{-1} Na_2HPO_4, 1.32 g l^{-1} KH_2PO_4, 1 g l^{-1} NH_4Cl, 1 ml l^{-1} Trace Elements A, and 1 ml l^{-1} Trace Elements B. Where indicated, 1 g l^{-1} yeast extract was also included. Trace Elements A consisted of 1 M $MgCl_2.6H_2O$ and 80 mmol l^{-1} $CaCl_2$. Trace Elements B contained 80 mmol l^{-1} $FeSO_4.7H_2O$, 20 mmol l^{-1} $ZnSO_4.7H_2O$, 20 mmol l^{-1} $MnSO_4.4H_2O$, 4 mmol l^{-1} $CuSO_4.5H_2O$, 4 mmol l^{-1} $CoSO_4.H_2O$, 4 mmol l^{-1} H_3BO_3, and 2% v/v concentrated HCl. Later experiments (as specified below) used more strongly buffered media, Minimal Medium 2 (MM2) containing 6 g l^{-1} Na_2HPO_4 and 3 g l^{-1} KH_2PO_4 (equivalent to M9 medium) with other components as for MM1. Chemicals were obtained from Sigma Aldrich, UK.

Cellulose degradation experiments used Ford's Gold Medal Blotting paper (2 cm squares, approx. 50 mg each), Whatman GB003 pure cellulose blotting paper (1 cm squares, approx 30 mg each), or Avicel (microcrystalline cellulose powder, Sigma-Aldrich 310697, 20 μm particles; 5g l^{-1}). In cultures lacking insoluble substrates such as filter paper or Avicel, growth was assessed by measurement of optical density at 600 nm. Such measurements were considered questionable where insoluble substrates

were present. Growth in these cases was assessed by serial dilution in sterile phosphate-buffered saline, followed by plating to LA without antibiotics and colony counting. During growth experiments with filter paper and Avicel, cultures were agitated vigorously on a vortex mixer for 60 seconds once every 24 hours. Growth was assessed by serial dilution and colony count.

Molecular biology procedures

Plasmids and BioBricks used in these experiments are shown in Table 1. Sequences of these BioBricks may be found in the Registry of Standard Biological Parts. PCR was performed using Kod Polymerase (Novogen) according to the manufacturer's protocol, except that for amplification of high GC genes from *C. fimi*, 10% v/v glycerol was included in reaction mixtures, and denaturation steps at the beginning of each cycle were extended to 1 minute. Genome sequencing of *C. freundii* SBS197 and NCIMB11490 was performed using the Ion Torrent instrument at the Centre for Bacterial Cell Biology, University of Newcastle. Detailed methods used for library construction, DNA purification and concentration check among others have been detailed in the supplementary information. Preliminary assessment of gene complement was performed using the RAST service (Aziz *et al*. 2008).

Construction of BioBrick cassettes

We chose to work with RFC10 BioBricks (Knight 2003) in making our constructs. At the time of designing constructs, relatively few assembly systems could allow flexible assembly of genes from a library prepared in a standard format (Ellis *et al*. 2011). Coding sequences of *cenA* and *cex* were amplified from *C. fimi* genomic DNA using primers (cenA-F -

CGTGAATTCGCGGCCGCTTCTAGATGTCCACCCGCAGAACC, cenA-R CGTTACTAGTATTATTACCACCTGGCGTTGC, cex-F CGTGAATTCGCGGCCGCTTCTAGATGCCTAGGACCACGCC and cex-R GCTACTAGTATTATTAGCCGACCGTGCAGG) with BioBrick RFC10 prefix and suffix, and cloned in pSB1A2 (Registry of Standard Biological Parts). Site directed mutagenesis was performed to remove a PstI restriction site, forbidden in BioBrick RFC10, from *cex* by alteration of codon 261 from CAG to CAA. Standard BioBrick assembly was used to add a strong synthetic ribosome binding site to each gene, and then to combine *cenA* and *cex*, and to add either a *lac* promoter for expression in *E. coli* or *C. freundii*, or *spac* promoter (Yansura and Henner 1984). BioBrick cassettes bearing a *spac* or *lac* promoter together with *cenA* and *cex* of *C. fimi* (Table 1) were prepared in pSB1A2 for testing in *E. coli* and *C. freundii.* The pSB1A2 constructs were introduced into *C. freundii* SBS197 generating strains SL0 (bearing pSB1A2-BBa_J33207, P_{lac}-*lacZ'*α), SL1 (bearing pSB1A2-BBa_15509, P_{lac}+*cenA*+*cex*), and SL2 (bearing pSB1A2-BBa_15510, P_{spac}+*cenA*+*cex*).

Cellulase activity assays

Qualitative assays were performed on Luria agar (LA) plates as described below whereas quantitative assays were performed either on culture medium or cell extracts. All cultures and experiments were performed in triplicates. Cell extracts were prepared from cell pellets using BugBuster® HT protein extraction reagent (Novagen, Inc) according to manufacturer's instructions. Qualitative assay of CenA activity was performed by growing cells on LA plates containing 0.1% w/v CMC. After overnight growth, plates were flooded with 0.1% w/v Congo Red, left for 15 min, poured away and then flooded with 1 M NaCl for 15 min. Areas where CMC was present were

297 stained red; areas where CMC had been hydrolysed were revealed as zones of
298 clearing. Quantitative assay of CenA was performed using CMC labelled with
299 Remazol Brilliant Blue (Azo-CMC, Megazyme) according to the manufacturer's
300 instructions. A calibration curve was prepared using different concentrations of RBB
301 under the same assay conditions and this was then used to determine the activity in
302 the test samples.
303
304 Cex activity was assayed using methylumbelliferyl-β-D-glucopyranoside (MUC,
305 Melford M1091). For qualitative assays, 0.1 ml MUC (5 mg/ml) was spread over agar
306 plates and allowed to dry prior to inoculation. Strains to be tested were then
307 inoculated and incubated overnight at 37°C. Following incubation, plates were
308 examined under UV illumination (364 nm) to reveal fluorescent methylumbelliferone
309 (4-MU) released by Cex activity. Alternatively, cells were suspended in buffer
310 (equivalent to MM2 medium base) containing 0.25 mg/ml MUC in a 1ml plastic
311 cuvette, and then examined under UV illumination. Quantitative assay of Cex was
312 performed in the same way, with methylumbelliferone detected using a fluorimeter
313 (Turner Biosystems Modulus single tube multimode reader, with UV fluorescence
314 module), and quantified with reference to a calibration curve.
315

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Note: parts of this project related to use of *E. coli* vector systems in *C. freundii* were presented at the International Genetically Engineered Machine competition (iGEM) 2012 by the University of Edinburgh student team, of which RN and JK were members.

Conflict of Interest

No conflict of interest declared.

SUPPORTING INFORMATION

Additional comments on growth conditions, figures of results as referred to in the main article

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Figures

Fig.1 Secretion of expressed cellulases by the different recombinant constructs of *E. coli* JM109 and *C. freundii* SBS197. Cells and culture supernatant were tested for exoglucanase and endoglucanase activity after 24 hours growth. A. Exoglucanase activity determined as the amount of 4-MU released per minute per ml; B. Endoglucanase activity determined using the Azo-CM cellulose assay (Megazyme Int., Ireland) based on release of Remazol Brilliant Blue.

Fig. 2 Growth with cellulose (filter paper or microcrystalline powder) as carbon source. Cultures contained MM1 as described in the text with 2 squares of 2×2 cm Fords Gold Medal Blotting paper, equivalent to 31 mmol l^{-1} glucose (A) or 100 mg microcrystalline cellulose (B). Results using *E. coli* JM109 constructs are presented in Supplementary Fig. 3.

Fig. 3 Effects of vortexing on filter paper break down, utilization and maintenance of cells. Growth conditions were as described for Fig. 2 above. Cells expressed CenA and Cex from the *spac* promoter (A) or the *lac* promoter (B). For 'V', cultures were vigorously agitated using a vortex mixer was for one minute every 24 hours, whereas for 'NV', no vortexing was performed. Area under the curves were calculated and compared (Supplementary Fig. 2a).

Tables

Table 1: Plasmids and BioBricks used in these experiments

Vector or BioBrick	Composition	Source
pSB1A2	pMB1 replication origin, ampicillin/carbenicillin resistance	Registry of Standard Biological Parts
pSB1C3	pMB1 replication origin, ampicillin/carbenicillin resistance	Registry of Standard Biological Parts
pSB2K3	F' replication origin plus P1 lytic replication origin, kanamycin resistance	Registry of Standard Biological Parts
pSB3C5	p15A replication origin, chloramphenicol resistance	Registry of Standard Biological Parts
pSB4C5	pSC101 replication origin, chloramphenicol resistance	Registry of Standard Biological Parts
pTG262 (modified for BioBrick use, BBa_I742123)	pWV01 replication origin, functional in both *E. coli* and *B. subtilis* (de Vos and Simons, 1994), chloramphenicol resistance	original plasmid kindly provided by C.A. Shearman and M.J. Gasson, Institute of Food Research, Norwich, UK.
BBa_J33207	*lac* promoter plus sequence encoding N-terminal 77 amino acids of LacZ	cloned from *Escherichia coli* BL21 genomic DNA
BBa_J15001	strong synthetic ribosome binding site	specifically designed
BBa_J15503	*spac* promoter	cloned from plasmid pVK168 kindly provided by Prof. Patrick Piggot, Temple University, Arizona

BBa_J15504	*lacI* encoding *lac* repressor	cloned from *Escherichia coli* BL21 genomic DNA
BBa_J15505	synthetic ribosome binding site plus *lacI*	Assembly of BioBricks shown above
BBa_K118023	*cenA* encoding endoglucanase	*Cellulomonas fimi* ATCC484
BBa_K118022	*cex* encoding bifunctional xylanase/exoglucanase (PstI site removed by silent mutation)	*Cellulomonas fimi* ATCC484
BBa_J15507	synthetic ribosome binding site plus *cenA*	Assembly of BioBricks shown above
BBa_J15508	synthetic ribosome binding site plus *cex*	Assembly of BioBricks shown above
BBa_J15506	*spac* promoter plus *lacI*	Assembly of BioBricks shown above
BBa_J15509	*lac* promoter plus RBS-cenA plus RBS-cex	Assembly of BioBricks shown above
BBa_J15510	*spac* promoter plus lacI plus RBS-cenA plus RBS-cex	Assembly of BioBricks shown above

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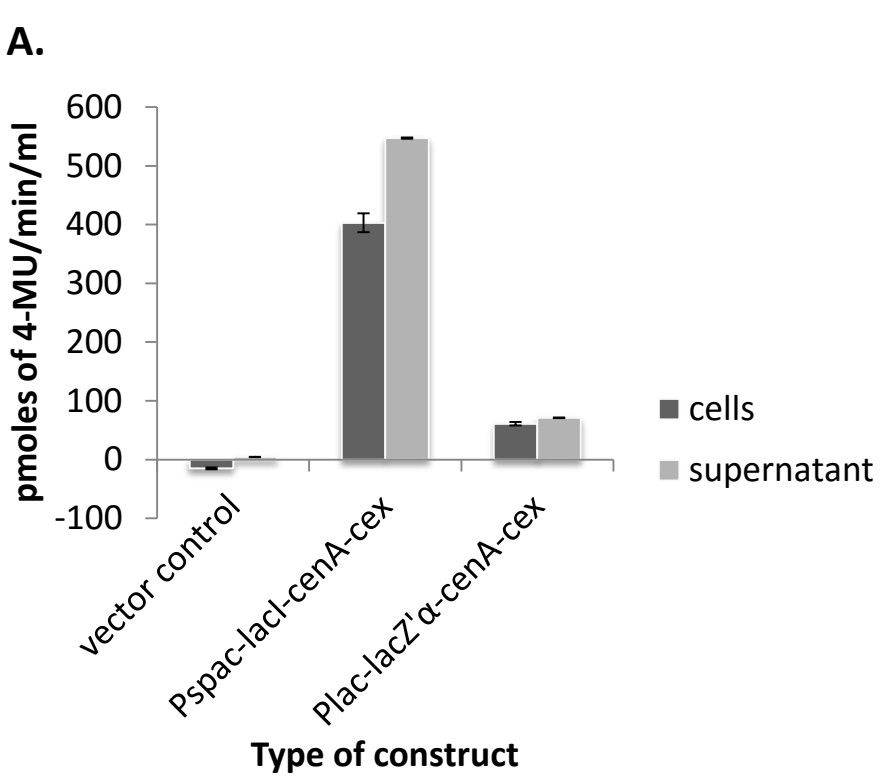
Figure 1


A.
pmoles of 4-MU/min/ml
600
500
400
300
200
100
0
-100
vector control
Pspac-lacI-cenA-cex
Plac-lacZ'α-cenA-cex
Type of construct
cells
supernatant

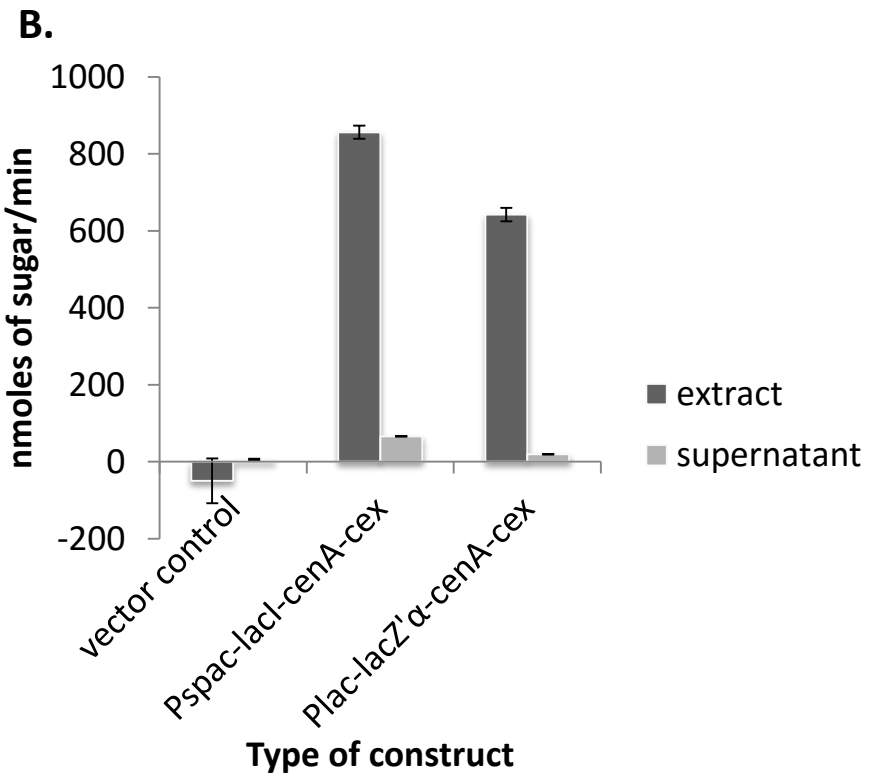

B.
nmoles of sugar/min
1000
800
600
400
200
0
-200
vector control
Pspac-lacI-cenA-cex
Plac-lacZ'α-cenA-cex
Type of construct
extract
supernatant

Figure 2

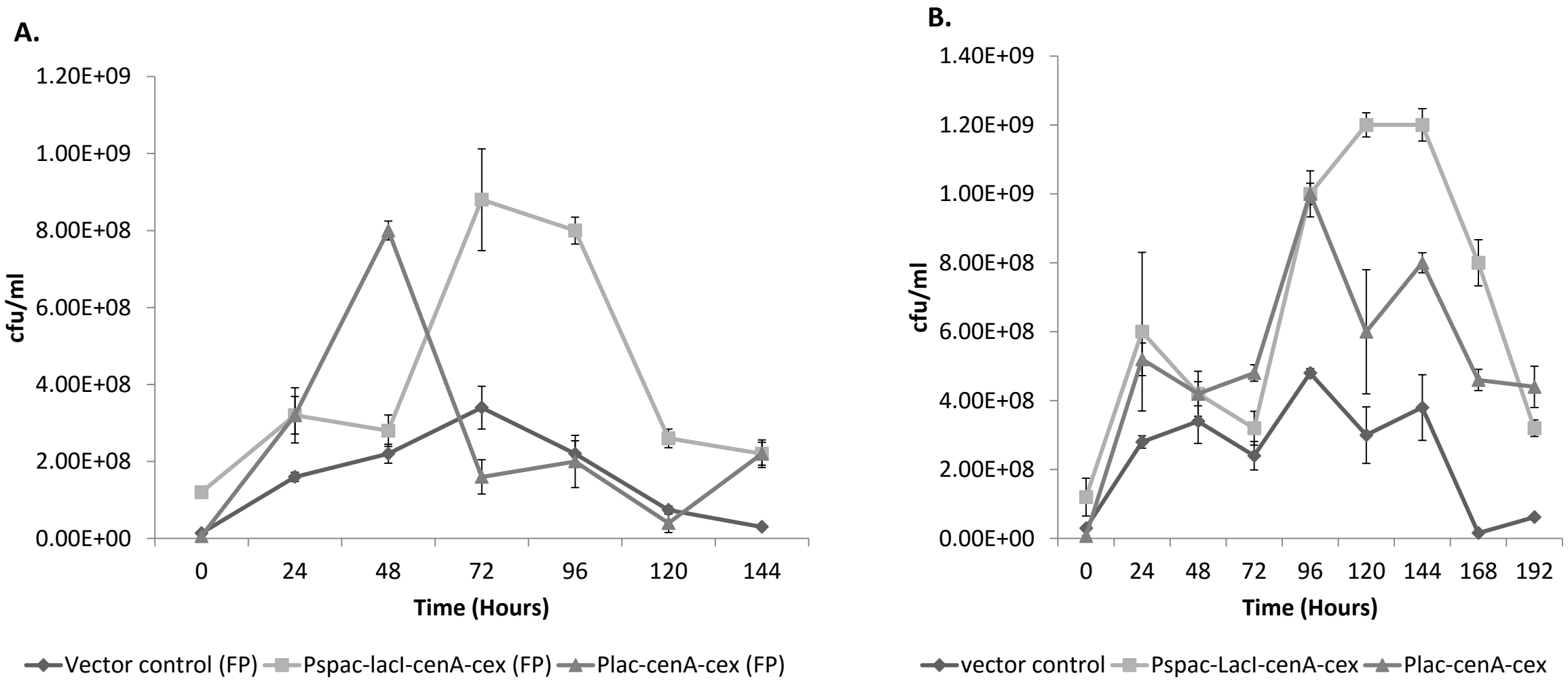
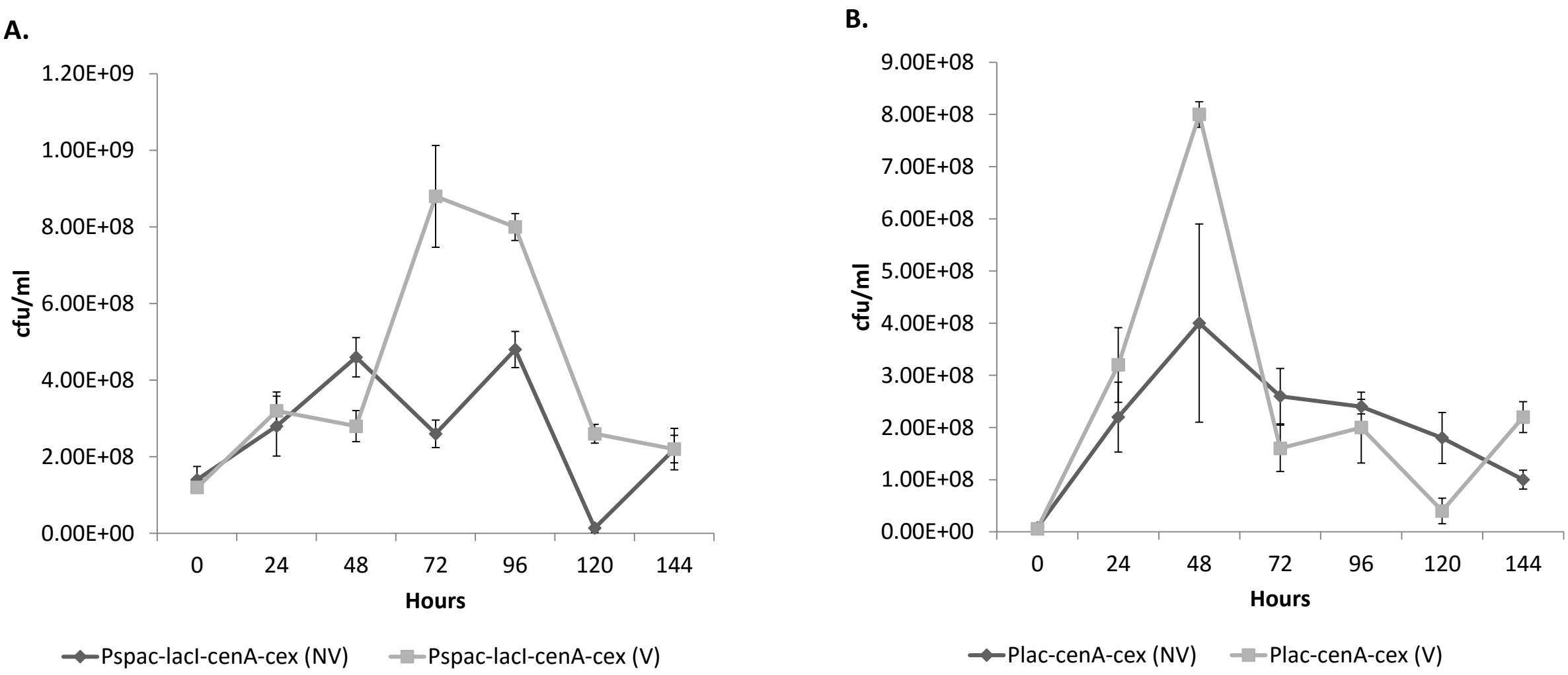

A.
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
cfu/ml
0
24
48
72
96
120
144
Time (Hours)
Vector control (FP)
Pspac-lacI-cenA-cex (FP)
Plac-cenA-cex (FP)
B.
1.40E+09
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
cfu/ml
0
24
48
72
96
120
144
168
192
Time (Hours)
vector control
Pspac-LacI-cenA-cex
Plac-cenA-cex

Figure 3


A.
1.20E+09
1.00E+09
8.00E+08
6.00E+08
4.00E+08
2.00E+08
0.00E+00
cfu/ml
0
24
48
72
96
120
144
Hours
Pspac-lacI-cenA-cex (NV)
Pspac-lacI-cenA-cex (V)
B.
9.00E+08
8.00E+08
7.00E+08
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
cfu/ml
0
24
48
72
96
120
144
Hours
Plac-cenA-cex (NV)
Plac-cenA-cex (V)