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Improved growth media and culture techniques for genetic analysis and assessment of biomass utilization by *Caldicellulosiruptor bescii*

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Abstract

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Methods for efficient growth and manipulation of relatively uncharacterized bacteria facilitate their study and are essential for genetic manipulation. We report new growth media and culture techniques for *Caldicellulosiruptor bescii*, the most thermophilic cellulolytic bacterium known. A low osmolarity defined growth medium (LOD) was developed that avoids problems associated with precipitates that form in previously reported media allowing the monitoring of culture density by optical density at 680 nm (OD_{680}) and more efficient DNA transformation by electroporation. This is a defined minimal medium and does not support growth when a carbon source is omitted, making it suitable for selection of nutritional markers as well as the study of biomass utilization by *C. bescii*. A low osmolarity complex growth medium (LOC) was developed that dramatically improves growth and culture viability during storage, making it a better medium for routine growth and passaging of *C. bescii*. Both media contain significantly lower solute concentration than previously published media, allowing for flexibility in developing more specialized media types while avoiding the issues of growth inhibition and cell lysis due to osmotic stress. Plating on LOD medium solidified by agar results in ~ 1,000-fold greater plating efficiency than previously reported and allows the isolation of discrete colonies. These new media represent a significant advance for both genetic manipulation and the study of biomass utilization in *C. bescii*, and may be applied broadly across the *Caldicellulosiruptor* genus.

Keywords

Caldicellulosiruptor; Biomass utilization; Defined growth media

Introduction

Caldicellulosiruptor species are capable of growing on a variety of unpretreated plant biomass materials, including poplar, switchgrass, Arabidopsis, and Napier grass [29] and are the most thermophilic cellulolytic bacteria known [3]. There are nine reported species in the *Caldicellulosiruptor* genus [5, 11, 13, 16–20, 22, 30], and eight have published sequenced genomes [4, 10, 14, 23]. All species show physiological differences, including different growth properties on different biomass substrates. Interestingly, while all species grow on xylan and acid-pretreated switchgrass, growth on crystalline cellulose is variable [2]. This suggests a variety of capabilities and the possibility of engineering strains for specific or even general use. Genomic analysis has been an invaluable tool for the prediction of genes involved in biomass utilization [2, 9, 23] showing that *Caldicellulosiruptor* species each produce dozens of glycosyl hydrolases [4, 25] and the genes predicted to be important are organized in clusters [9], making mutational analysis to identify potential key genes relatively straightforwardly. Recently developed genetic tools [6, 7] will, in fact, allow those predictions to be tested directly. One of the greatest barriers to both genetic selection and the analysis of biomass utilization has been the absence of a truly defined minimal medium. Previous media [7, 8, 29] were not suitable for nutritional selection of transformants and plating efficiencies were extremely low, making detection of transformants problematic. Cells formed diffuse colonies that were difficult to isolate. During the course of developing methods for genetic analysis of *Caldicellulosiruptor* species, we developed new media that not only allowed nutritional selection but also eliminated precipitates that interfered with assessment of cell growth. The new media resulted in significantly improved growth, and

more efficient and reliable transformation. Because this is a truly defined medium, it does not support growth without an added carbon source making it suitable for the study of lignocellulosic biomass degradation and utilization. We also present refined plating methods that give greater plating efficiency with the ability to streak for isolated colonies. These technical advancements represent a significant step forward in the ability to apply genetic methods to *Caldicellulosiruptor* species, and to the ability to study biomass utilization by *Caldicellulosiruptor* species.

Materials and methods

Caldicellulosiruptor bescii strains and media

Modified DSMZ 640 medium [29], and a defined variant of this medium that lacks yeast extract and casein [7], has been described for the growth of *C. bescii*. For liquid media, a 1 % inoculum was used and cells were grown for 16–20 h at 75 °C, unless otherwise specified.

Initial liquid growth media contained sulfide and were prepared with mineral solution [8], trace element solution SL-10 [26], vitamin solution [28], 19 amino acid solution [1], 0.25 mg/l resazurin, 2.4 mM KH_2PO_4, and 5 g/l maltose. The media were prepared aerobically, 2 g/l $NaHCO_3$ was added, and the mixture was reduced using 3 g/l cysteine HCl and either 1 g/l Na_2S (as used previously), 0.5 g/l Na_2S or no Na_2S. The pH was adjusted to 6.8, and the media were aliquoted into serum bottles, sealed, and degassed by four cycles of vacuum and argon. The final pH after degassing was 7.2. Wild-type *C. bescii* was inoculated into 50-ml cultures and culture density was compared after 12 and 24 h of growth. Liquid growth media without sulfide were prepared with mineral solution [8], trace element solution SL-10 [26], vitamin solution [28], 0.25 mg/l resazurin, 2.4 mM KH_2PO_4, and 5 g/l maltose. In separate batches, the media were prepared with 19 amino acid solution [1], 0.05 % (w/v) casein hydrolysate, or both. The media were prepared aerobically, 2 g/l $NaHCO_3$ was added, and the mixture was reduced using 3 g/l cysteine HCl. The pH was adjusted to 6.8, and the media were aliquoted into serum bottles, sealed, and degassed by four cycles of vacuum and argon. The final pH after degassing was 7.2. Wild-type *C. bescii* was inoculated into 50-ml cultures and culture density was compared after 12 and 24 h of growth. Liquid growth media without sulfide, casein, or amino acids but varying the phosphate concentrations, were prepared with a modified mineral solution (containing 0.14 g/l $CaCl_2 \times 2H_2O$ but the same amounts of other salts), trace element solution SL-10 [26], vitamin solution [28], 0.25 mg/l resazurin, and 5 g/l maltose. These media were prepared with 2.4 mM, 1 mM, 100 μM, 10 μM, 1 μM, or no KH_2PO_4. The media were prepared aerobically, 2 g/l $NaHCO_3$ was added, and the mixture was reduced using 3 g/l cysteine HCl. The pH was adjusted to 6.8, and the media was aliquoted into serum bottles, sealed, and degassed by four cycles of vacuum and argon. The final pH after degassing was 7.2. Wild-type *C. bescii* was inoculated into 50-ml cultures and culture density was compared after 12 and 24 h of growth. Liquid growth media without sulfide, casein, or amino acids, with phosphate reduced to 10 μM were prepared with a modified mineral solution (containing 0.14 g/l $CaCl_2 \times 2H_2O$ but the same amounts of other salts), trace element solution SL-10 [26], vitamin solution [28], 0.25 mg/l resazurin, 100 μM KH_2PO_4, and 5 g/l maltose. The media were prepared aerobically, 2 g/l $NaHCO_3$

was added, and the mixture was reduced using 3 g/l cysteine HCl. In a separate batch, 1 g/l $NaHCO_3$ was added, and the mixture was reduced using 1 g/l cysteine HCl. The pH was adjusted to 6.8, and the media were aliquoted into serum bottles, sealed, and degassed by four cycles of vacuum and argon. The final pH after degassing was 7.2. Wild-type *C. bescii* was inoculated into 50-ml cultures and culture density was compared after 12 and 24 h of growth.

LOD and LOC media were prepared from filter sterilized stock solutions. The 50× CbeI partial base salt solution contains 16.5 g of $MgCl_2$, 16.5 g of KCl, 12.5 g of NH_4Cl, 7 g of $CaCl_2 \times 2H_2O$, and 0.68 g of KH_2PO_4 per liter. The 1,0009 trace element SL-10 is prepared as described [26], and the vitamin solution is prepared as described previously [28], except concentrated to 2000×. Resazurin is dissolved in water to a concentration of 5 mg/ml. Uracil is dissolved in water to a concentration of 20 mM. Maltose, yeast extract, and casein hydrolysate are dissolved in water to a concentration of 10 % (w/v). LOD liquid medium contains 1× CbeI partial base salts, 1× trace minerals, 1× vitamins, 0.25 mg/l resazurin, and 5 g/l maltose, and when necessary, 40 μM uracil. Biomass media contain all these components, except that maltose is omitted, and biomass is added prior to bottling, to a concentration of 5 g/l. Transformation media contain all the same components as defined media, with the addition of 19 amino acid solution. LOC medium contains all the same components as LOD medium, with the addition of 1 g/l yeast extract and 2 g/l casein hydrolysate. Water used for preparing media was boiled and cooled to drive off dissolved oxygen. Media were reduced with 1 g/l cysteine HCl, buffered with 1 g/l, $NaHCO_3$, pH adjusted to 6.8 with 8 M NaOH, and degassed with argon. The final pH is 7.2. Solid media were prepared as described for liquid media, but the media was prepared as a 2× concentrate. This is added to an equal volume of 3 % agar solution (for a final 1.5 % agar) prior to pouring. Cells were either plated onto the solidified agar surface, then overlaid with 4 ml of 1.5 % agar, or suspended in the overlay and poured onto the plate surface.

Monitoring cell growth

Cell density was monitored by cell count using a Petroff-Hausser counting chamber and phase contrast microscope with 40× magnification and expressed as cells/ml. Optical density was monitored using a Jenway Genova spectrophotometer, measuring absorbance at 680 nm.

Results and discussion

Previous versions of growth media produced insoluble precipitates

Precipitates in the growth media complicate a number of protocols, including transformation by electroporation, DNA isolation, and the assessment of cell growth directly by measuring optical density as opposed to cell counts, which are time-consuming and laborious. There are a variety of published media for growth of *Caldicellulosiruptor* species and our initial studies used previously published media, modified DSMZ 640 [29], (Table 1) containing a trace element mix that included aluminum and EDTA [1]. A precipitate formed during the preparation of this mix, and the reason for including aluminum was not obvious. A previously developed trace element mix, Trace Element SL-10 [26], used in both the

DSMZ516 and DSMZ640 medium, does not include aluminum and EDTA and does not form any precipitate during preparation. For this reason, the trace elements solution previously used in modified DSMZ 640 (Table 1) was replaced with Trace Element SL-10 (Table S1).

In the preparation of previously published media, including DSMZ516 and DSMZ640, a white precipitate formed after the media components were combined. To address this, we decreased the amounts of $CaCl_2$ and KH_2PO_4. Decreasing the amount of $CaCl_2$ from 0.33 g per liter to 0.14 g per liter had no discernable effect on growth. This was not surprising, since DSMZ 640 medium contains no added calcium. Apparently, any calcium requirement is met by the trace amounts from yeast extract. We prepared media with varying phosphate concentrations, ranging from 0 to 2.4 mM present in the original DSMZ 516 medium to no phosphate and used a 1 % inoculum from a culture grown with 2.4 mM phosphate. All cultures, including those with no phosphate, grew well after the first passage but because there was likely carryover of phosphate and/or actively dividing cells from the inoculum, cells were subcultured in media with reduced phosphate concentrations. As shown in Fig. 1c, media containing 10 μM phosphate resulted in a slight reduction in growth. An amount of 100 μM phosphate was sufficient to support full growth and passage of cells ten times in 100 μM phosphate showed full growth (data not shown). These reduced calcium and phosphate concentrations resulted in a media formulation that does not form white precipitates, even when prepared as a 2× concentrate for preparation of solid media for plating.

In DSMZ 516 and DSMZ 640 media, phosphate was used as a pH buffer, however, $NaHCO_3$ and acetate provide effective pH buffering during growth. With a pKa of 6.4, there is sufficient bicarbonate to stabilize pH during early growth. *C. bescii* produces acetate (pKa = 4.8) during growth, which actually becomes the predominant buffering agent during late-phase growth. This bicarbonate/acetate buffer system smoothly buffers the pH change from the initial pH of 7.2 down to ~5.0, which we have found to be the pH after full growth in batch culture LOD medium.

In addition, cell pellets contained a black precipitate and we postulated that this resulted from the presence of sodium sulfide in the media used as a reductant. Removing sodium sulfide from the media entirely actually resulted in significantly better growth (Fig. 1a) and eliminated the black precipitate in cell pellets.

The LOD formulation also avoids a number of technical problems. The formation of black and white precipitates was problematic for transformation by electroporation, and transformation is more reproducible in the absence of these precipitates (data not shown). Plasmid purification protocols were more efficient and more reliable using cells grown in LOD medium. The precipitates also made monitoring growth by optical density unreliable. The white precipitate, which was most likely calcium phosphate or magnesium phosphate (which has a tenfold lower solubility than calcium phosphate), appeared in uninoculated or early growth-phase media, and the black precipitate, started to accumulate in cultures during log phase. These precipitates interfered with measurements of OD, resulting in falsely high OD measurements during growth. The absence of these precipitates in our reformulated

LOD medium allowed reliable monitoring of growth by OD. We typically use the red light wavelength 680 nm because resazurin, a redox indicator and a component of LOD medium, has a broad absorption spectrum through the visible range, but no measurable absorbance at OD_{680}. Cell density correlates linearly with OD_{680}, with $OD_{680} = 1$ corresponding to 10^9 cells/ml (Fig. 2).

Reformulated LOD medium has a reduced solute concentration

One of the most striking features of LOD medium is the low total solute concentration compared to previous media formulations. In the course of making numerous media refinements, several components were either removed or reduced (Table 1). Removing most of these components did not affect growth, and removing sulfide actually had a beneficial effect, making LOD better than previous media for growth. We speculated that one of the reasons for the improved growth was lower osmolarity in the media. To test this, we assayed defined modified DSMZ 640 medium, and our new LOD medium for NaCl tolerance. In defined modified DSMZ 640 medium, NaCl was inhibitory with as little as 20 mM added NaCl (~190 total milliosmolar (mOsm)), and nearly complete growth inhibition was observed with 50 mM NaCl (~250 total mOsm) (Fig. 3a). In LOD medium, NaCl tolerance was significantly higher, but tolerance of total osmolarity was comparable. Growth was nearly normal with 50 mM added NaCl (~170 total mOsm), and inhibition was observed similarly at higher osmolarities (Fig. 3b). These results support the notion that a major growth advantage to LOD medium is due to a reduction in total solute concentration.

The sensitivity of *C. bescii* growth to solute concentration is consistent with what has been previously determined in *C. saccharolyticus*, for which growth is inhibited at a total solute concentration of 0.200 Osm/kg [15], and cell lysis is induced above 0.218 Osm/kg [27]. Our previous defined modified DSMZ 640 medium [7] contained an initial total solute concentration of at least 0.151 Osm/kg calculated from the defined components, and not accounting for additional yeast extract and casein. The fermentation of maltose to lactate, CO_2, and H_2 during growth increases the total osmolality in the culture media, potentially to inhibitory levels. Our new LOD medium contains an initial total solute concentration of 0.074 Osm/kg, with no complex components unaccounted. The decrease in total solute concentration also gives greater flexibility in adding additional components to the growth media, which would otherwise be inhibitory due to osmolality. This solute flexibility will be advantageous in processes that require the addition of additional components or the accumulation of products during growth.

LOD and LOC media result in comparable growth to previously published media

Previously published media contained amino acids, yeast extract, and casein, or a combination of these substrates. We found that casein and/or yeast extract enhanced growth significantly and that removal of amino acids had no significant effect on growth whether or not casein was included (Fig. 1b). The growth benefit of peptides, but not amino acids, may reflect an inability of *C. bescii* to take up single amino acids, while peptides may be transported more efficiently.

In comparison to other reported *Caldicellulosiruptor* media, defined modified DSMZ 640 [7] contained more $NaHCO_3$ and cysteine HCl. Reducing the amount of $NaHCO_3$ by half and cysteine HCl threefold to 1 g/l had no effect on either growth rate or final cell density (Fig. 1D). We previously reported that defined modified DSMZ 640 was adjusted to a final pH of 6.8 [7]. We point out that while the pH is adjusted to 6.8 prior to bottling and degassing, the final pH is 7.2.

While LOD medium supports good growth, the addition of yeast extract and casein dramatically improved growth rate as well as cell survival during storage. For these reasons, we chose to add yeast extract and casein to our LOD medium, a formulation that we call low osmolarity complex (LOC) medium, for some purposes. As shown in Fig. 4, *C. bescii* grows well in either LOD or LOC medium, reaching a final cell density from a 0.1 % inoculum in 24 and 18 h, respectively. Growth in LOC medium was faster, and the final optical density was slightly greater (~0.27 for LOC compared to ~0.22 for LOD). During exponential growth, the generation time was approximately 2 h in LOC medium and 4 h in LOD medium.

A 1 % inoculum is convenient for overnight growth, but there is carryover of nutrients and/or grown cells, resulting in some detectable cell growth even in the absence of a carbon source. Serial passaging or decreasing the inoculum to 0.1 % avoids this problem. Maltose is used as a carbon source in our defined medium. As expected of a minimal medium, omitting maltose as carbon source in LOD medium resulted in no detectable growth (OD_{680} = 0.001 ± 0.001), even after 24 h. However, LOD without maltose, but with added casein (2 g/l) or yeast extract (0.5 g/l), grew to OD_{680} = 0.007 ± 0.002 and OD_{680} = 0.022 ± 0.002, respectively. These cultures reached this density in as little as 12 h. It was previously reported [29] that modified DSMZ 640 without maltose did not support growth. The presence of the precipitates in this medium makes slight growth difficult to detect. In the investigation of generating a truly defined medium, we observed that the addition of the amounts of yeast extract or casein used in modified DSMZ 640 medium did in fact support some growth without an additional carbon source. Furthermore, the addition of yeast extract stimulates growth on poor carbon sources such as biomass. We suggest that LOD medium is more suitable for the assessment of biomass utilization since cells growing on this medium with biomass as the only carbon source are utilizing the biomass exclusively for growth. Using LOD medium for assessment of growth on biomass, we have found that *C. bescii* grows well on switchgrass (as previously reported), however, growth on poplar is poor and final cell density is an order of magnitude less and on the order of 10^7 cells/ml (Chung, in preparation).

Plating efficiency, genetic selections, and transformation efficiency are dramatically affected by media composition

Genetic manipulation requires the ability of plate individual colonies and select transformants. Since we rely on a nutritional marker, uracil prototrophy, for selection a defined minimal media is essential. We previously reported using 1.0 % Phytagel to solidify plating media and soft agar overlays. Phytagel requires divalent cations to solidify [21], making it a poor gelling agent for use with low-salt media. Even with the addition of

additional Ca^{+2} and Mg^{+2}, the soft agar overlays never fully solidified during incubation, resulting in diffuse colonies that were difficult to pick cleanly. This also required that plates be incubated agar side down, causing condensation to drip onto the plate surface also making the isolation of discrete colonies difficult. We determined that 1.5–1.8 % agar can be used to solidify LOD medium for plating, and that this formulation remains solid during incubation at 75 °C. Surprisingly, we also found that plating with agar instead of Phytagel resulted in a nearly a 1,000-fold increase in plating efficiency. This increase may be related to the calcium and magnesium concentrations or availability in the solid media. We attempted to grow *C. bescii* on an agar surface without the soft agar overlay, but never observed growth. Increasing the agar overlay concentration to 1.5 % produced an overlay that remained solid, and could be incubated inverted avoiding dripping on the agar surface from condensation. Using 1.5 % agar overlays also resulted in the formation of discrete colonies that are smaller and well separated from each other on plates. In addition, and of real importance for genetic manipulation, cells may be streaked for isolation on an agar surface and then covered with an agar overlay allowing the isolation and purification of mutants. We found that LOD medium works well for selection of uracil prototrophs, and growth of auxotrophic mutants can be supported by the addition of 20 mM uracil, as previously reported [7]. The increased plating efficiency eliminates the need to plate large numbers of cells, which often results in background growth during prototrophic selections and allows for detection of transformants at lower frequency.

DNA transformation is significantly more efficient and reliable with LOD medium, though amino acid mix is required in the defined media for preparation of competent cells for transformation. We observed in early experiments that success in transformation corresponded to the use of early log phase cells, before the appearance of a black color in the cell pellet. In LOD medium, no such precipitate forms and transformation works reliably when the competent cell culture grows to an optical density between 0.13 and 0.16 with an optimum at 0.15. Plasmid isolation protocols are also more reliable and efficient when cells are grown in LOD medium (Chung, manuscript in preparation).

Conclusions

Here we report the development of growth media and culture techniques that improve growth, eliminate precipitates, and make our media suitable for genetic manipulation and the analysis of biomass utilization. One of the most striking features of our new media is the total solute content. LOD medium contains fewer solutes per liter compared to either DSMZ 516 or 640 recipes and it has been well documented that *C. saccharolyticus*, for example, is exceptionally sensitive to solute concentration [15, 24, 27], likely accounting for some of the growth advantage in our new media.

Removal of Na_2S had the most obvious benefit to growth, most likely due to sulfide toxicity, as long lag times due to sulfide were observed in the closely related *Clostridium thermocellum* [12]. Decreasing the amount of cysteine in the media to 1 g/l, which is more in range of DSMZ medium 640 [22], was sufficient for reducing the media, and keeping it anaerobic during growth.

Transformation by electroporation is more reliable in LOD, likely because there are no precipitates, which may interfere with electroporation and the range of cell densities that work for electroporation is larger. It is not clear why amino acids are required for transformation but the addition of glycine, a common cell wall weakening agent in bacterial transformations, in the mixture may weaken the cell wall making electroporation more efficient.

LOD medium will be especially useful for analyzing the ability of *Caldicellulosiruptor* species to grow on biomass. As shown in Fig. 2, modified DSMZ 640 [29] does support some growth, even in the absence of an added carbon source. Plant biomass of 5 g/l does support growth in LOD medium and the growth rate varies depending on the type of biomass, allowing a true assessment of biomass utilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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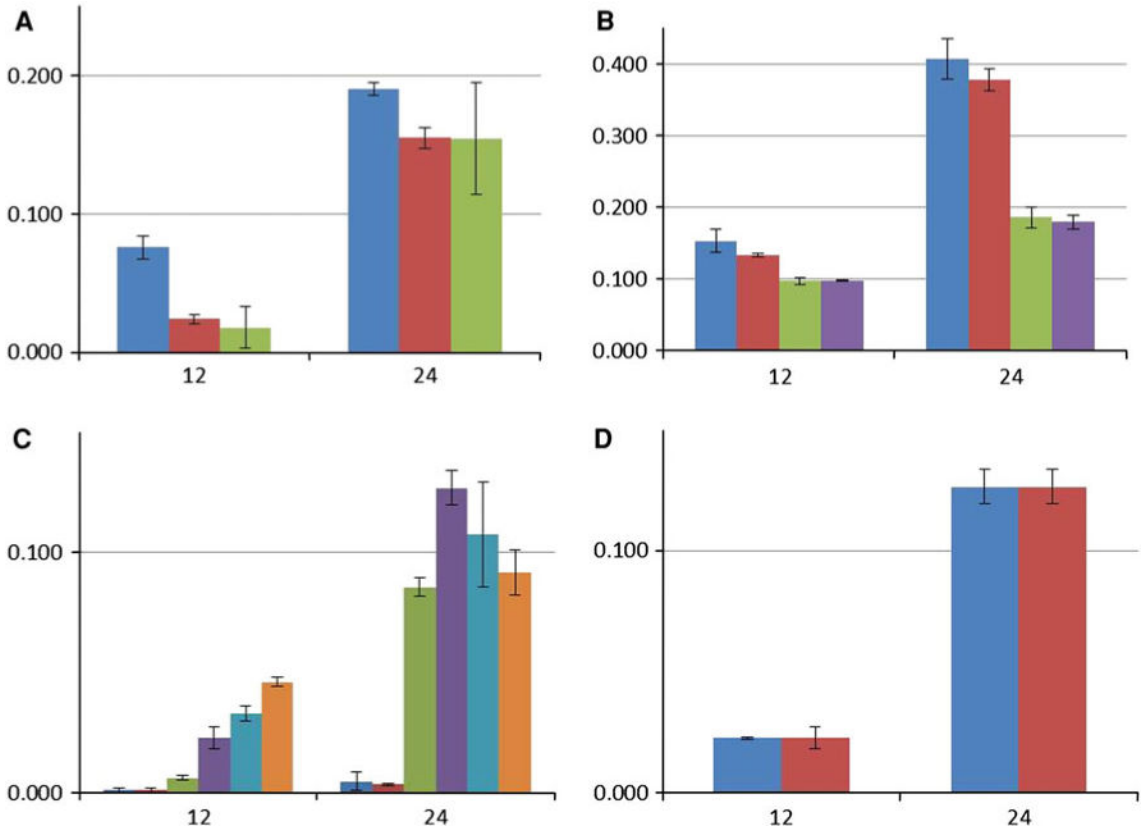

Fig. 1.

Growth of *C. bescii* in various media measured by optical density at 680 nM. **a** Modified defined DSMZ 640 medium with 0 g/l (*blue*), 0.5 g/l (red), or 1 g/l (*green*) sodium sulfide. **b** Sulfide-free modified defined DSMZ 640 with casein and amino acids (*blue*), casein only (*red*), amino acids only (*green*), or neither (*purple*). **c** Defined modified DSMZ 640 without sulfide, casein, or amino acids with 0 μM (*blue*), 1 μm (*red*), 10 μM (*green*), 100 μM (*purple*), 1 mM (*aqua*), or 2.4 mM (*orange*) phosphate. **d** Modified defined DSMZ 640 medium without sulfide, casein, or amino acids, 10 μM phosphate and 11.9 mM sodium bicarbonate, 5.7 mM cysteine (*blue*) or 23.8 mM sodium bicarbonate, 17.1 mM cysteine (*red*). Error bars represent standard deviation and each point is the average of three replicates

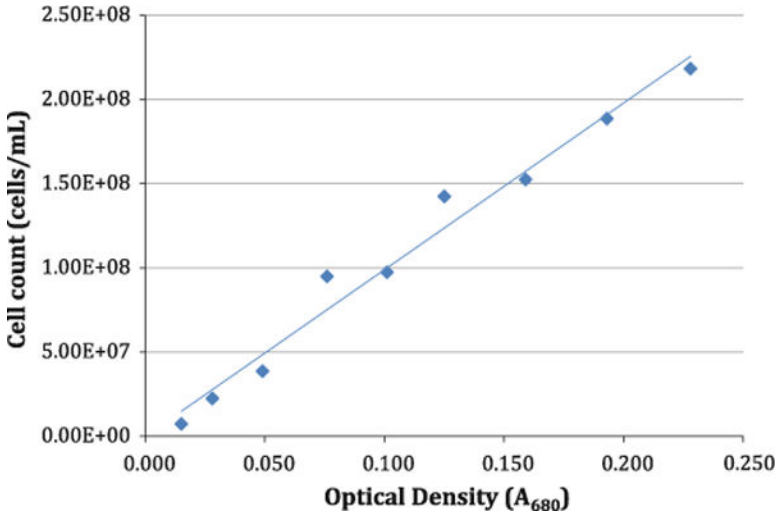

Fig. 2.

Relationship between cell number and OD_{680}. Cell concentration is linearly related to OD_{680}. OD = 1 corresponds to a cell density of 10^9 cells/ml. $R^2 = 0.977$

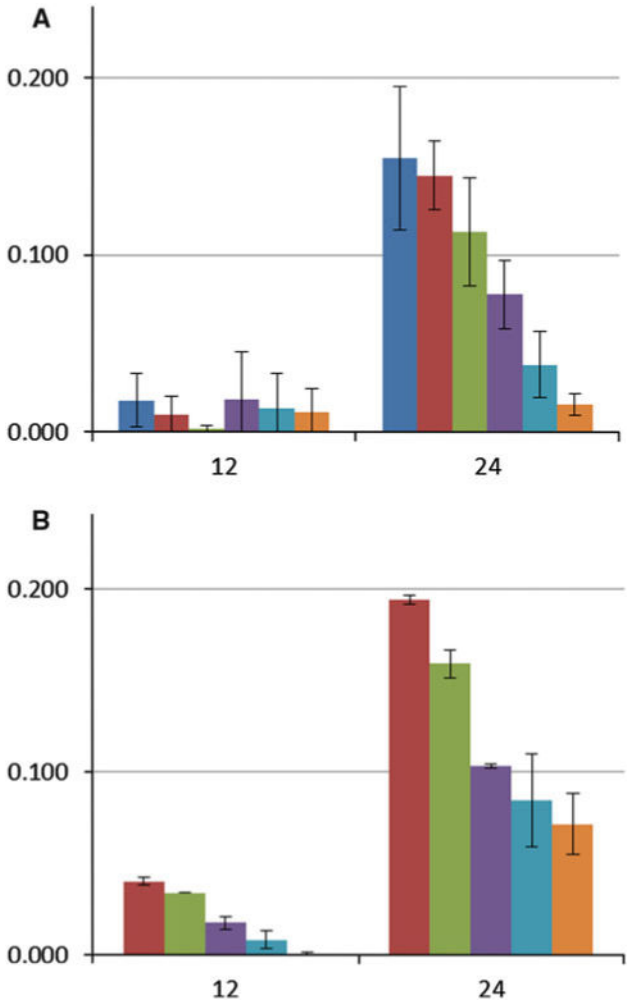

Fig. 3.

NaCl tolerance in defined modified DSMZ 640 [7] and LOD media. Growth measured by optical density at 680 nM. **a** Defined modified DSMZ 640 (~150 mOsm), without added NaCl (*blue*), and with NaCl added to ~170 mOsm (*red*), ~190 mOsm (*green*), ~210 mOsm (*purple*), ~230 mOsm (*aqua*), and ~250 mOsm (*orange*). **b** LOD medium with NaCl added to ~170 mOsm (*red*), ~190 mOsm (*green*), ~210 mOsm (*purple*), ~230 mOsm (*aqua*), and ~250 mOsm (*orange*). Error bars represent standard deviation and each point is the average of three replicates

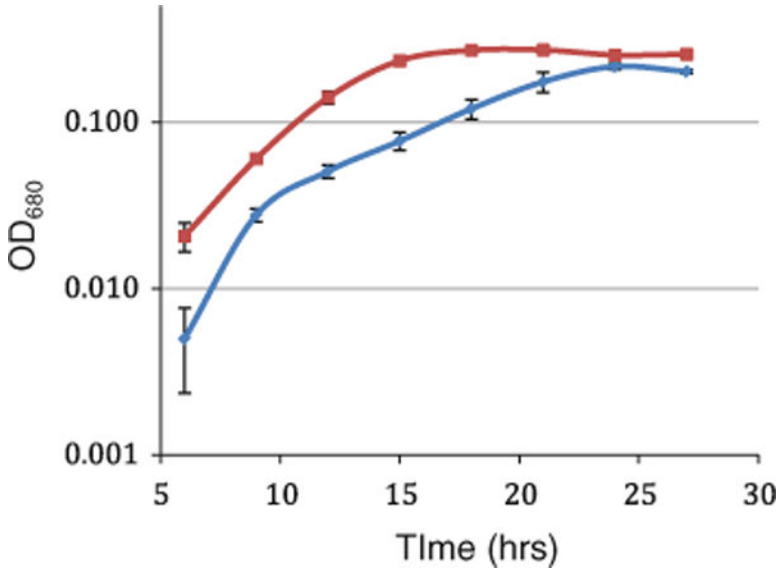

Fig. 4.

Growth of *C. bescii* in low osmolarity defined (LOD) media (blue) and low osmolarity complex (LOC) media (*red*). Error bars represent standard deviation and each point is the average of three replicates

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Table 1

Comparison of media compositions

	DSMZ 516		DSMZ 640		Modified DSMZ 640 [29]		Defined modified DSMZ 640 [7]		LOD		LOC	
	g/l	mM	g/l	mM	g/l	mM	g/l	mM	g/l	mM	g/l	mM
Salts												
NH_4Cl	0.33	6.17	0.9	16.83	0.33	6.17	0.25	4.67	0.25	4.67	0.25	4.67
NaCl	–	–	0.9	15.40	–	–	–	–	–	–	–	–
KH_2PO_4	0.33	2.42	0.75	5.51	0.33	2.42	0.33	2.42	0.0136	0.10	0.0136	0.10
K_2HPO_4	–	–	1.5	8.61	–	–	–	–	–	–	–	–
KCl	0.33	4.43	–	–	0.33	4.43	0.33	4.43	0.33	4.43	0.33	4.43
$MgCl_2 \times 6H_2O$	0.33	1.62	0.4	1.97	0.33	1.62	0.33	1.62	0.33	1.62	0.33	1.62
$CaCl_2 \times 2H_2O$	0.33	2.24	–	–	0.33	2.24	0.33	2.24	0.14	0.95	0.14	0.95
$NaHCO_3$	1.5	17.86	–	–	1.00	11.90	2	23.81	1	11.90	1	11.90
$Na_2S \times 9H_2O$	0.5	2.08	–	–	0.5	2.08	1	4.16	–	–	–	–
Total salt*	3.65	36.83	4.45	48.32		30.87	4.57	43.36	2.0636	23.68	2.0636	23.68
Ions												
NH_4^+		6.17		16.83		6.17		4.67		4.67		4.67
Na^+		22.02		15.40		16.07		27.97		15.90		15.90
K^+		6.85		22.73		6.85		6.85		4.53		4.53
Ca^{+2}		2.24		0.00		2.24		2.24		0.95		0.95
Mg^{+2}		1.62		1.97		1.62		1.62		1.62		1.62
Cl^-		18.33		36.16		18.33		16.84		14.25		14.25
$H_2PO_4^-$		2.42		14.12		2.42		2.42		0.10		0.10
HCO_3^-		17.86		0.00		11.90		23.81		11.90		11.90
HS^-		2.08		0.00		2.08		4.16		0.00		0.00
Total ions*		79.60		107.21		67.70		90.59		53.93		53.93
pH	7.1–7.3		7.2		7.2		6.8		7.2		7.2	
Trace elements	SL-10 [26]		SL-10 [26]		Trace Minerals [1]		Trace Minerals [1]		SL-10 [26]		SL-10 [26]	
Organics												
Maltose/cellobiose	5	14.60	1	2.90	5.00	14.60	5	14.60	5	14.60	5	14.60

	DSMZ 516		DSMZ 640		Modified DSMZ 640 [29]		Defined modified DSMZ 640 [7]		LOD		LOC	
	g/l	mM	g/l	mM	g/l	mM	g/l	mM	g/l	mM	g/l	mM
Cysteine HCl × H_2O	–	–	0.75	4.27	0.5	2.85	3	17.08	1	5.69	1	5.69
Yeast extract	0.5	nd	1	nd	0.5	nd	–	–	–	–	1	nd
Trypticase	–	–	2	nd	–	–	–	–	–	–	2	nd
Amino acid mix	–	–	–	–	–	–	3.58	28.76	–	–	–	–
[Total organics]		14.60		7.17		17.45		60.44		20.29		20.29
Total mOsm/l*		94.20		114.38		85.14		151.04		74.23		74.23

*Total salt and ion calculation does not include trace minerals or vitamins. LOD and LOC are pH adjusted by the addition of 4 mM NaOH, which is included in these calculations. Additional acid/base for adjusting pH of other media is not included in these calculations. Osmolarity of complex organic substrates like casein and yeast extract are not determined (nd) and also not included in total osmolarity