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Extracellular production of L-ascorbic acid by *Chlorella protothecoides*, *Prototheca* species, and mutants of *P. moriformis* during aerobic culturing at low pH

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Nine strains of *Chlorella protothecoides* and 43 strains representing the five species of *Prototheca* were screened in flask culture for their ability to synthesize L-ascorbic acid (AA). Ascorbic acid was detected in all strains, ranging from 4.8 to 0.38 mg AA g^{-1} of dry cells. Organisms selected for further study grew well and maintained their AA productivity above a pH of 3.5. They can produce AA using a variety of carbon and nitrogen sources. Aerobic fermentation of selected strains resulted in extracellular accumulation of AA up to 76 mg l^{-1}. By classical mutagenesis and selection methods, we created mutants of *Prototheca moriformis* ATCC 75669 that produced greater quantities of AA than the wild-type strain (78.4 *vs* 21.9 mg AA g^{-1} of cells). A process based on extracellular production could greatly reduce the cost of AA manufacture by eliminating the need for extraction of the AA from the cells.
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Introduction

L-ascorbic acid (AA; vitamin C) is a water-soluble vitamin essential for the growth and nutrition of humans and other animals [28]. Currently, most AA is produced commercially by variations of the Reichstein process, which entails one bacterial fermentation and six chemical synthesis steps [5,16,23]. We had previously developed a one-step fermentation process using the heterotrophic green microalga *Chlorella pyrenoidosa* [26]. That process combined strain improvement efforts to increase the organism's productivity through classical mutagenesis techniques, and liquid culture studies to optimize the process in controlled fermentors. The program improved the intracellular AA content more than 70-fold, and resulted in cells containing more than 2% of their dry weight as AA [8]. However, virtually none of the AA in the above process was extracellular. AA is highly oxygen-labile at a pH greater than 7 [16,32]. Extracellular production would greatly ease the recovery process for large-scale commercial production. We describe the initial screening of 52 strains of *C. protothecoides* and *Prototheca* for AA accumulation, and an aerobic fermentation process for the production of AA in which the AA accumulates extracellularly. The stabilization of AA in the aerobic environment is made possible by culturing these organisms under acidic conditions. Classical mutagenesis of *P. moriformis* ATCC 75669 has resulted in mutants that produce substantially greater quantities of AA than the wild-type (wt) strain. The results of some of these works are described in US Patents 5,792,631 and 5,900,370 [24,25].

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Materials and methods

Microbial strains

Strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA), the Culture Collection of Algae and Protozoa at the University of Texas at Austin (UTEX), or from Prof. R. Scott Pore (West Virginia University Medical School, Charleston, WV).

Media

Table 1 lists the formulations of the media for growth and maintenance of the strains. Slant medium was the standard plate medium containing 2.5 g L^{-1} yeast extract. Unless otherwise indicated, all chemicals were reagent grade, purchased from Sigma (St. Louis, MO). Glucose for fermentors was calculated on an anhydrous basis, and supplied as glucose monohydrate (AE Staley Manufacturing, Decatur, IL).

Initial screening of strains

All organisms were cultured axenically. Initial screening of *C. protothecoides* and *Prototheca* strains was done in 50 ml of ATCC medium no. 5 in 250-ml baffled shake flasks, incubated at 21°C on a rotary shaker at 160 rpm. ATCC medium no. 5 contains (per liter): yeast extract, 1 g; beef extract, 1 g; tryptose, 2 g; ferrous sulfate, trace; glucose, 10 g; pH 7.2. Aliquots for total whole broth AA were extracted for 15 min with an equal volume of 5% trichloroacetic acid (TCA). Supernatant samples were analyzed for AA by high-pressure liquid chromatography (HPLC) directly after centrifugation at 5000×*g* for 5 min.

Mutagenesis

Mutant isolates were generated by treatment of cell suspensions with nitrous acid, ethyl methane sulfonate, or ultraviolet light.

Table 1 Media for growth and maintenance of strains[a]

Ingredient	Liquid		Agar	
	Standard	Mg-limiting	Ferrozine plates	Standard plates
Potassium phosphate monobasic	1.3	1.3	0.27	2.0
Potassium phosphate dibasic	3.8	3.8	1.4	2.0
Trisodium citrate, dihydrate	7.7	7.7	1.3	2.6
Magnesium sulfate heptahydrate	0.40	0.02	none	0.40
Ammonium sulfate	3.7	3.7	1.0	1.0
Trace metals solution (no copper)[b] (ml)	2	2	2	2
Ferrous sulfate heptahydrate (mg)	1.5	4.5	1.5	1.5
Calcium chloride dihydrate	–	0.25	–	–
Manganous sulfate monohydrate	–	0.08	–	–
Added after autoclaving				
Copper sulfate pentahydrate, 100 g/l (ml)	–	–	2	–
Ferrozine, 40 g/l, in 5 mM phosphate (pH 7.5 final) (ml)	–	–	8.8	–
Ferric ammonium sulfate dodecahydrate, 40 g/l (ml)	–	–	3.8	–
Glucose	40	60	10	10
Thiamine HCl (mg)	2	3	1	1

[a]All amounts are in grams per liter, except where noted. The pH of all media was 7.2 before autoclaving. Plate media was solidified with 1.5% agar.
[b]Metal concentrations (mg/l) in TM stock, added as: calcium chloride dihydrate, 3102; manganous sulfate monohydrate, 400; cupric sulfate monohydrate, 0.8 (fermentor medium only); cobalt chloride pentahydrate, 40; boric acid, 160; zinc sulfate heptahydrate, 400; sodium molybdate dihydrate, 19; vanadyl sulfate, 20; nickel nitrate hexahydrate, 8; sodium selenite, 16.

Glucose-depleted cells grown in standard liquid medium were washed by centrifugation and resuspension in 25 mM phosphate buffer, pH 7.2, and diluted to approximately 10^7 colony-forming units (CFU)/ml. Cells in suspension were exposed to the mutagen to achieve about 99% kill, resuspended in standard liquid medium, and incubated 4–8 h in the dark. Portions of cell suspensions were spread to obtain isolated colonies on standard agar medium or agar medium containing ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; see below).

Mutant screening

The mutant screening program evaluated isolated mutant colonies first on the original isolation plates after mutagenesis, then in small-scale liquid tube culture, then in shake flask culture, and finally in controlled fermentors. All incubations were done at 35°C. Although designed to isolate strains that produced increased amounts of AA, occasionally strains were isolated, which produced less AA than their parent strains. Some mutant colonies on standard agar medium were picked randomly and subcultured to master plates. Other isolation plates were inverted over chloroform to lyse cells on the surface of the colonies, releasing AA. Released AA was detected by spraying the treated plates with a solution of 2,6-dichrorophenol-indophenol (1.25 g l^{-1} in 70% EtOH), a blue redox dye which AA reduces to its colorless form [20]. Colonies derived from mutagenized cells *via* either method were saved to master plates for further evaluation, and in the second case if their clear halos were significantly larger than the halos typical of the other mutant colonies in that group. Other mutagenized cells were spread onto plates containing ferrozine and ferric iron (Table 1). Ascorbic acid reduces ferric ion to ferrous ion; ferrous ion forms a violet complex with ferrozine [19]. Colonies giving the darkest violet color reactions were master-plated. During the course of these platings, some colonies appeared much lighter than the surrounding colonies. These strains were saved for testing and many of them produced much less AA than their parent strains.

For primary screening of tube cultures, cells were inoculated from master plates into 4 ml of Mg-limiting medium in 16× 125 mm tubes, and the tubes were shaken in a slanted position on a rotary shaker at 300 rpm for 4 days. After both 3 and 4 days of incubation, aliquots were removed for AA assay and cell density determination. Those for AA assay were centrifuged at 1500×*g* for 5 min and the resulting supernates were removed for either colorimetric or chromatographic analysis. Promising isolates were retested in tube culture. Those showing increased AA production in the tube screen were tested in shake flasks.

For secondary screening of flask cultures, cells were inoculated into 50 ml of standard Mg-sufficient flask medium in 250-ml baffled shake flasks, and incubated on a rotary shaker at 180 rpm until glucose depletion (24–48 h). A second series of standard flask medium was inoculated from the first set to a cell density of $0.15A_{620}$, and incubated for 24 h. A series of Mg-limiting flask medium was inoculated from the second set by a 1/50 dilution and incubated for 96 h. Flasks were sampled periodically for AA analysis and cell density measurements.

For dry weight determinations of cell density, 5-ml whole broth samples were centrifuged at 5000×*g* for 5 min, washed once with distilled water, and the pellet was washed into a tared aluminum weighing pan. Cells were dried for 8–24 h at 105°C. Dry cell weight was calculated by difference.

AA analysis

For colorimetric analysis of AA, a modification of the dipyridyl method of Omaye *et al* [20], adapted to 96-well microplates, was used. Whole culture broth was extracted with TCA as above. Twenty-five-microliter aliquots of culture supernates or whole broth extracts were added to microplate wells, followed by addition of 125 μl of color reagent. The color reagent consisted of four parts 0.5% 2,2′-dipyridyl and one part 8.3 mM ferric ammonium sulfate in 15% (vol/vol) *o*-phosphoric acid; the two components were mixed immediately before use. After 1 h, the absorbance at 520 nm was read. AA concentration was calculated by comparison with the absorbances of AA standards.

High-pressure liquid chromatographic analysis was based on the method of Grun and Loewus [10]. Samples were assayed on a

Bio-Rad HPX-87H organic acid column (Bio-Rad Laboratories, Richmond, CA) with 13 mM nitric acid as solvent, at a flow rate of 0.8 ml min^{-1}. Detection was at 254 nm. This system can distinguish between the L- and D-isomers of AA (9.92 *vs* 10.35 min retention times).

Fermentation

For fermentation, 14-l vessels (New Brunswick Scientific, New Brunswick, NJ) were filled with 6 l of 5.5 mM potassium phosphate, pH 7.0, and autoclaved. After sterilization, 20 ml of glucose salts solution was added. This was prepared by autoclaving separately concentrates of the following compounds, and combining them afterwards to achieve the concentrations listed (mM): trisodium citrate, 21.8; magnesium sulfate, 30.7; sulfuric acid, 165; sodium phosphate 20; ferrous sulfate, 0.36; glucose, 2600; trace metals solution, 9.4 ml (see Table 1 footnote); thiamine HCl, 25 mg l^{-1} (filter-sterilized). Each fermentor was equipped with a variable-speed impeller for agitation, a dissolved oxygen probe, a pH probe with control system, a sterile air input, a temperature control system, and various ports for aseptic additions and sampling. pH was

Table 2 Results of initial strain screening in flasks[a]

Strain name	Strain number	Cell dry weight (g l^{-1})	Whole broth AA (mg l^{-1})	Specific formation (mg AA g cells^{-1})	Growth rate (h^{-1})
C. protothecoides	ATCC 75667	6.6	8.0	1.2	0.17
	UTEX 25	6.2	7.5	1.2	0.09
	UTEX 29	7.0	4.6	0.64	0.08
	UTEX 31	3.4	1.3	0.38	0.06
	UTEX 249[b]	10.8	12.2	1.1	0.14
	UTEX 250	9.2	7.6	0.83	0.04
	UTEX 255	5.2	2.5	0.48	0.03
	UTEX 256	6.6	3.6	0.55	0.05
	UTEX 411	7.0	7.4	1.1	0.09
P. moriformis	ATCC 75669	10.4	19.2	1.8	0.19
	BTR 1080	7.8	17.0	2.2	0.03
	BTR 1181	10.0	16.8	1.7	0.15
	UTEX 288	12.2	16.8	1.4	0.17
	UTEX 1439	6.8	4.6	0.68	0.18
P. stagnora	UTEX 1443	9.0	25.6	2.8	0.04
P. ulmae	BTR 1275[b]	8.6	10.0	1.2	0.05
	BTR 1277	10.1	6.3	0.62	0.11
P. wickerhamii	BTR 972	7.8	5.9	0.76	0.18
P. zopfii	ATCC 16527	6.5	6.2	0.95	0.15
	ATCC 209695	7.8	26.8	3.4	0.13
	ATCC 209696	7.8	37.4	4.8	0.15
	BTR 870	10.2	8.6	0.84	0.19
	BTR 871	7.8	13.8	1.8	0.18
	BTR 879	8.6	14.4	1.7	0.19
	BTR 893	7.1	8.3	1.2	0.15
	BTR 899	8.2	25.2	3.1	0.08
	BTR 946	9.8	20.6	2.1	0.17
	BTR 948	7.1	8.5	1.2	0.15
	BTR 1002	12.9	12.8	0.99	0.16
	BTR 1034	9.8	13.2	1.4	0.19
	BTR 1043	9.2	16.8	1.8	0.17
	BTR 1078	10.2	19.1	1.9	0.17
	BTR 1110	10.8	12.7	1.2	0.18
	BTR 1140	13.0	20.1	1.6	0.16
	BTR 1145	12.8	19.2	1.5	0.19
	BTR 1177	12.5	26.2	2.1	0.17
	BTR 1183	9.4	16.0	1.7	0.19
	BTR 1205	14.8	30.0	2.0	0.33
	BTR 1213	7.6	11.7	1.5	0.25
	BTR 1218	12.0	29.4	2.4	0.17
	BTR 1250	9.8	9.1	0.93	0.16
	BTR 1254	9.7	17.4	1.8	0.22
	BTR 1263	9.5	22.6	2.4	0.18
	BTR 1334	13.6	9.2	0.68	0.14
	BTR 1367	14.4	11.7	0.81	0.21
	BTR 1368	9.2	19.4	2.1	0.11
	BTR 1369	12.8	11.2	0.88	0.19
	BTR 1403	11.6	13.7	1.2	0.12
	UTEX 178	10.8	26.0	2.4	0.15
	UTEX 329	6.2	10.7	1.7	0.14
	UTEX 1434	8.6	18.1	2.1	0.16
	UTEX 1438	11.7	18.4	1.6	0.17

[a]Strains incubated in 50 ml of ATCC medium no. 5 in 250-ml baffled flasks at 21°C on a rotary shaker at 180 rpm.
[b]A total of 2.5 g l^{-1} yeast extract added to growth medium.

controlled by addition of anhydrous ammonia. Unless otherwise noted, fermentations were controlled at 35°C. Before inoculation, agitation was set at 300 rpm, air at 0.1 l min^{-1}, and the pH was brought to the desired set point with anhydrous ammonia. Fermentors were inoculated with actively growing cultures to an initial cell density of about 0.3 g cells l^{-1} (dry weight basis).

Results

Initial screening of strains

Promising preliminary shake flask and fermentor work with *Prototheca zopfii* UTEX 1438 prompted us to test in flasks a wide variety of *Prototheca* strains and strains of *C. protothecoides*. Table 2 shows the results of the initial flask screening. AA was detected in all 52 strains. Strains of *P. moriformis*, *P. wickerhamii*, and *P. zopfii* grew best, with 38 of 40 strains having growth rates greater than 0.1 h^{-1}. Seven of the nine strains of *C. protothecoides* had growth rates of less than 0.1 h^{-1}. Strains produced between 0.33 and 4.8 mg AA g^{-1} of dry cells. Cells of most of the *P. zopfii* strains aggregated in liquid culture and adhered to the sides of flasks, making microbiological manipulations difficult. This trait is the result of the lack of the polysaccharide capsule (R. Scott Pore, personal communication). Based on this trait, growth rate, AA production, cell yield on glucose, and ability to grow at elevated temperatures, *P. zopfii* BTR 1254, *P. moriformis* ATCC 75669, and *C. protothecoides* ATCC 75667 were chosen for further work. In particular, controlled fermentors were used to assess their abilities to synthesize AA under conditions more closely resembling those in an industrial fermentation process.

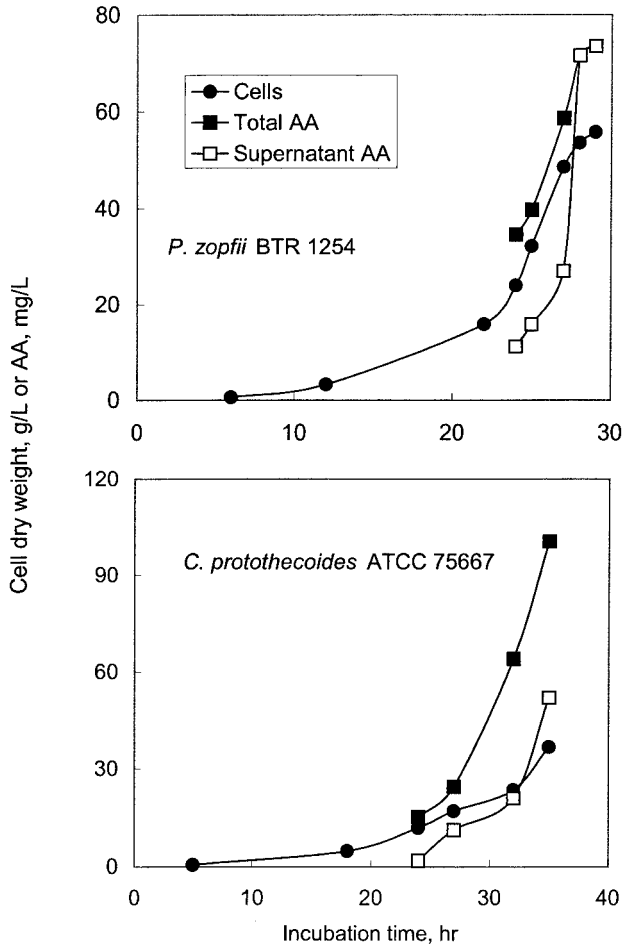

Figure 1 Fermentation of *P. zopfii* BTR 1254 and *C. protothecoides* ATCC 75667 in 14-l aerated vessels. After 18 h of incubation, the pH set point was lowered from 5.0 to 4.0 (*P. zopfii*) and from 5.0 to 3.5 (*C. protothecoides*).

Table 3 Growth and AA production of *P. moriformis* ATCC 75669 under various culture conditions in flasks[a]

Variable	Ending pH	Cell dry weight (g l^{-1})	Whole broth AA (mg l^{-1})	Specific formation (mg AA g^{-1} cells)
Temperature, °C				
25	4.8	10.6	41.4	3.9
30	5.0	12.6	51.9	4.1
35	4.7	11.3	40.0	3.5
Starting pH				
7.2	4.7	11.3	40.0	3.5
5.8	5.3	10.6	36.7	3.5
4.5	3.3	13.2	41.6	3.2
Nitrogen source				
Ammonium	4.7	11.3	40.0	3.5
Urea	7.2	1.4	1.9	1.4
Glutamate	4.8	11.3	61.2	5.4
Casamino acids	6.6	11.5	47.3	4.1
Carbon source				
Glucose	4.7	11.3	40.0	3.5
Fructose	5.3	7.7	30.8	4.0
Glycerol	5.3	9.4	43.2	4.6
Ethanol	5.3	8.4	37.9	4.5
n-Propanol	4.6	2.0	6.7	3.4

[a]All strains incubated in 50 ml of standard liquid medium in 250-ml baffled flasks on a rotary shaker at 180 rpm. Except where indicated, carbon and nitrogen sources were glucose and ammonium, respectively, temperature was 35°C, and the starting pH was 7.2.

Fermentation

Follow-up 14-l fermentations at 30°C were done with *P. zopfii* BTR 1254 and *C. protothecoides* ATCC 75667 to test their ability to grow and produce AA at low pH. Carbon and nitrogen sources were glucose and ammonium, respectively. Figure 1 illustrates the progress of the fermentations. In the *P. zopfii* fermentation, the pH set point was lowered from 5.0 to 4.0 after 22 h of incubation. By the end of the run, the extracellular and whole broth concentrations of AA were the same (76 mg l^{-1}), indicating that the cells were freely permeable to AA and, at pH 4, extracellular AA is stable in the presence of measurable dissolved oxygen.

In the *C. protothecoides* fermentation, the pH set point was lowered from 5.0 to 3.5 after 18 h of incubation. The overall growth rate was 0.16 h^{-1}. At the end of the run, the extracellular AA level was about half that of the whole broth level, suggesting that this organism is not as permeable as *P. zopfii* to AA.

Testing in flasks

Although strain BTR 1254 performed well in the early phases of testing, it exhibited an unacceptably high rate of spontaneous conversion to the aggregating cell type mentioned above. For subsequent work, the similar strain *P. moriformis* ATCC 75669 was chosen. It was grown under a variety of conditions in flasks. Table 3

summarizes the results of culture with varying temperatures, starting pH, nitrogen sources, and carbon sources. On glucose (30 g l^{-1}) and ammonium (25 mM), neither the temperature (25–35°C) nor the starting pH (4.5–7.2) had a significant effect on cell yield or final AA levels. Substitution of urea for ammonium greatly reduced both cell yield and AA formation, while substitution of glutamate for ammonium increased AA productivity somewhat. Growth of cells on ethanol or glycerol yielded results typical of glucose-grown cells. Growth on *n*-propanol significantly reduced cell yield, but had little effect on AA on a per-cell basis. These results demonstrate the versatility of this organism to produce AA under a variety of cultural conditions and with various nutrients.

Specific AA formation by P. moriformis mutant strains

The strain *P. moriformis* ATCC 75669 was selected as the parent strain in a classical mutagenesis strain improvement effort to isolate mutants that produced increased or decreased levels of AA. Figure 2 is a family tree showing the genealogy of each strain and shows the relative AA-synthesizing abilities of each under Mg-limiting incubation (see below). The best mutants produced nearly four times more AA than did the parent strain. A number of mutants were isolated with reduced specific AA formations, and some made none at all.

Effect of magnesium on AA synthesis

Although the wt strain ATCC 75669 showed only a modest increase in AA accumulation under Mg-limiting conditions relative to Mg-sufficient conditions, mutants that overproduce AA (e.g., ATCC PTA-111) showed significant increases in AA accumulation when Mg-limited (Figure 3). The effect in the mutant occurred at a cell density which was half that of the density needed for the effect to be manifest in the wt strain. The cause of this Mg effect has yet to be determined. In these strains, there was no significant difference between supernatant and whole broth concentrations of AA.

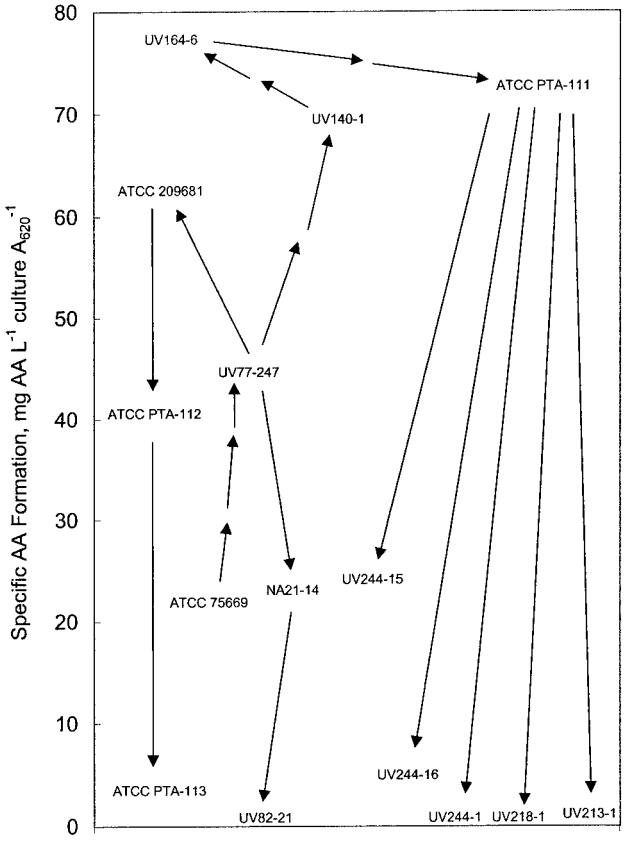

Figure 2 Genealogy of selected isolates. Specific AA formations of cells grown in magnesium-limiting shake flask culture for 44–48 h.

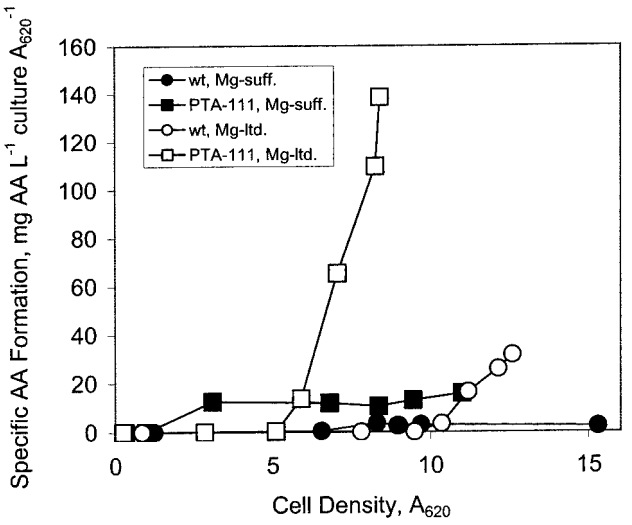

Figure 3 Effect of Mg on supernatant AA accumulation in flask cultures of the wt (ATCC 75669) and a high AA-producing strain (ATCC PTA-111) of *P. moriformis*.

Discussion

Members of the genus *Prototheca* are related to members of the genus *Chlorella* [13,17,21], especially *C. protothecoides* [15]. Both are placed in the microalgal class Chlorophyceae, but *Prototheca* species do not have photosynthetic pigments [22]. They have been called colorless *Chlorellas* [4,36], but this is somewhat misleading in that they have other characteristics that clearly distinguish them from members of most *Chlorella* species: (1) they cannot use nitrate as a sole source of nitrogen [21,22]; (2) they are all thiamine auxotrophs [22]; (3) they are acidophilic [14]; and (4) they can use a wide variety of hydrocarbons as sole sources of carbon [18,35,36]. *C. protothecoides* shares most of these traits, and is the species of *Chlorella* that is the most closely related to the genus *Prototheca* [6], particularly *P. wickerhamii* (R.S. Pore, personal communication).

We developed a process for AA production by aerobic fermentation of the organism *C. pyrenoidosa*, but the AA accumulated intracellularly, with little or none of it detectable in the culture supernate [26]. The major obstacle to the development of an aerobic one-step fermentation process for the production of AA is its oxygen lability. *C. pyrenoidosa* cannot grow below pH 5, and when we attempted to culture it at pH values low enough to begin to stabilize the AA in the aerobic environment (below pH 6), its AA productivity fell precipitously (data not shown).

A process exists for the extracellular production of the D-isomer of AA, erythorbic acid [29,34,38]. That process uses the filamentous fungus *Penicillium*, and titers of D-erythorbate reached 80 g l^{-1}. The study showed that erythorbate could be preserved in the supernate in an aerobic fermentation by controlling the culture

pH near 4.0. We have identified a group of acidophilic microalgae, *Prototheca* sp., related to *Chlorella* that grow well above pH 3.5 and maintain their AA productivity under these acidic conditions in an aerobic environment. Moreover, the extracellular AA level was 50–100% of the total whole broth concentration.

Whether by chemical synthesis [16,23], by the bacterial conversion of sorbitol [27,33], sorbose [30,39] or sorbosone [30], or glucose through 2,5-diketo-D-gluconic acid [1,5,9,31], existing or proposed commercial AA synthesis pathways rely on the production of 2-keto-L-gulonic acid (2-KLG). All of these, in turn, rely on the conversion of 2-KLG into AA by esterification and lactonization [3,7,11,23]. More recent work has focused on the biological conversion of 2-KLG to AA [5,12]. A biological process for the one-step conversion of glucose to AA would circumvent the requirements for such chemical conversion and greatly simplify the process. There has been little discussion in the literature concerning such a one-step process, presumably because of the problem of the rapid degradation of AA in an oxygenated environment. We describe here such a one-step process, in which the AA accumulates extracellularly in an acidic oxygenated fermentation culture. What remains is the demonstration of productivities high enough to make such a one-step process economically feasible. A process based on extracellular production could greatly reduce the cost of AA manufacture by eliminating the need for extraction of AA from the cells. Now that the AA biosynthetic pathway in plants is known [2,37], the achievement of this goal may be possible through rational genetic techniques. Although theoretically further improvements to the process could be made using gene shuffling and directed evolution, not all of the genes in the proposed pathway have been isolated, and no genetic system exists for *Prototheca*.

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