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ORIGINAL RESEARCH PAPER

Production of imidazole alkaloids in cell cultures of jaborandi as affected by the medium pH

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Abstract The effect of pH (from 4.8 to 9.8) on the production of pilosine and pilocarpine and on their partition between cell and medium was studied in two lineages (P and PP) of *Pilocarpus microphyllus* cell suspension cultures. Highest mass accumulation was observed at high pHs and both lineages produced pilocarpine while only lineage PP produced pilosine. Both alkaloids were released in the medium but higher accumulation occurred in the cells. The highest production of pilocarpine was at pH 8.8–9.8 in both cell lineages. Other imidazole alkaloids were also identified in both lineages. At all pHs tested, the pH in the media cultures tended to stabilize around 6 after 10–15 days of cultivation. NO_3^- and NH_4^+ variation in the media might partially explain the pH stabilization.

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Introduction

Pilocarpine is an imidazole alkaloid used in the treatment of glaucoma, as a stimulant of sweat and lachrymal glands, and in the context of xerostomia (Wynn 1996; Migdal 2000; Davies et al. 2001). Although more efficient synthetic substances have been produced, such as beta-blockers, pilocarpine is still the standard substance for the treatment of glaucoma.

Plants of the genus *Pilocarpus* (Rutaceae) are the only source of pilocarpine in nature and jaborandi (*P. microphyllus*) is the species accumulating the highest concentration of this alkaloid in its leaves (0.5–1%) (Pinheiro 1997). Although information on the content of pilocarpine in jaborandi is available (Pinheiro 1997; Andrade-Neto et al. 1996), very little is known about its biosynthesis (Avancini et al. 2003; Abreu et al. 2005, 2007a, b; Sandhu et al. 2006; Sawaya et al. 2008).

Because several secondary metabolites have physiological effects on animals, many of them are commercially exploited as components of medicines, insecticides, colorants and aromatizers (Verpoorte et al. 1999). Cell cultures have been intensively studied as an alternative to produce secondary

metabolites in a profitable manner by using large bioreactors (Verpoorte and Memelink 2002) and variations in the temperature, oxygen availability, light, medium composition and exposure to elicitors are usually evaluated in these studies (Dong and Zhong 2001; Biondi et al. 2004).

Previously, we have observed a strong influence of medium pH on the production of pilocarpine and the related alkaloid pilosine in calluses obtained from jaborandi leaf petioles (Abreu et al. 2005) and, later, we validated cell suspensions as a model to study the biosynthesis of imidazole alkaloids in jaborandi (Abreu et al. 2007a). In this work, two jaborandi cell lineages were studied regarding their ability to produce and release pilocarpine and pilosine in the medium, as well as the effects of pH on the partition of these alkaloids between cell and medium. Our long-term aim is to understand how pH affects the transport of the alkaloids through the membrane in order to get clues about the mechanisms governing the process.

Materials and methods

Cell cultures

Calluses obtained from jaborandi leaf petioles were used to establish cell suspension cultures (Abreu et al. 2007a). Two cell lineages (P and PP) were selected for this work due to their rapid growth rate and visual aspects, but mainly because they differed in the production and release of pilocarpine and pilosine in the culture medium, as shown by preliminary analyses (data not shown). These cell cultures were grown in 125 ml Erlenmeyer flasks with 30 ml MS culture medium (Murashige and Skoog 1962), containing 5.77 μM 2,4-D, and 3% sucrose (pH 5.8) and kept in the dark in an orbital shaker (110 rpm). Sub-culturing was carried out each 15 days.

Cell growth curve

Approx. 2 g cells from 15-day-old cell cultures were transferred to flasks containing media at varying pHs: 4.8, 5.8, 6.8, 7.8, 8.8 and 9.8. The cultures were maintained in darkness at 110 rpm agitation. A growth curve was obtained sampling 3 cultures flasks and determining the cell dry weight every 5th day starting at day 10. This experiment was repeated three times and, although some variations have been observed in terms of final cell mass, the final position of each treatment was the same. Therefore, only the results of the first experiment are presented.

Biochemical determinations

Cell cultures were established as above and allowed to grow for 30 days. They were used for two proposes: (1) During 12 days, on every second day, 300 μl of medium were collected from each flask and the pH was determined with a narrow pH electrode and then NO_3^- and NH_4^+ concentrations were determined in the medium according to Cataldo et al. (1975); McCullough (1967), respectively; (2) From the same flasks, 2 ml aliquots were collected every 5th day, starting at day 10, until the culture had completed 30 days. The cells were recovered by filtration, freeze-dried and then analysed for alkaloids. The pH of these 2 ml aliquots were also determined, and then the aliquots were freeze-dried for alkaloid analysis. We did not observe any salt precipitation in the media at any pH. This experiment was repeated twice with similar results regarding pH and alkaloid determinations. In the first experiment, however, we did not measure NO_3^- and NH_4^+ and therefore only the data of the second experiment is presented. Three replicates were set up for each pH treatment.

Extraction and analysis of pilocarpine and pilosine

Freeze-dried cells were extracted and quantified by HPLC according to Avancini et al. (2003). The media cultures were extracted by adding the solvents, following the same sequence as for freeze-dried cells. Injections with pure standards of pilocarpine (Sigma) and pilosine (a gift from the Centroflora Group—http://www.centroflora.com.br) were used to calculate the alkaloid concentration in the samples. Retention time, UV spectra and co-injection with pure standards were used to identify the substances in the chromatography runs. The concentrations were expressed as $\mu g\ g^{-1}$ cell dry mass and or $\mu g\ ml^{-1}$ culture medium.

Identification of alkaloids by mass spectrometry

Imidazole alkaloids in the cells or media were identified according with Abreu et al. (2005).

Results

Lineages P and PP differed markedly in terms of morphology. Lineage P formed friable and small cell clusters, while lineage PP formed bigger and compact cell clusters.

Both lineages showed the best cell growth at pHs 6.8 and 8.8 (Fig. 1) and a similar trend regarding pH variation. However, lineage P (Fig. 2a) stabilized at a lower pH than lineage PP (Fig. 2b).

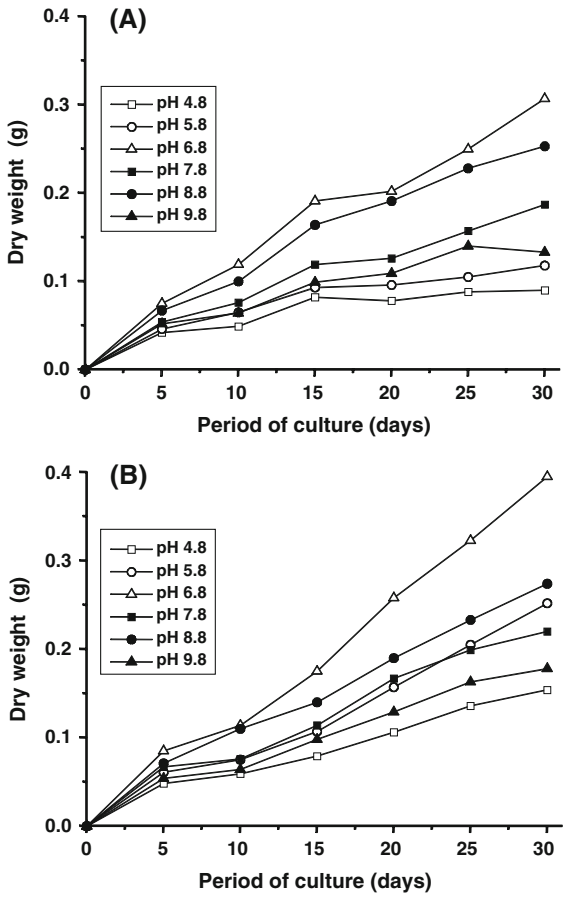

Fig. 1 Cell mass accumulation in cell suspension cultures of lineages P (**a**) and PP (**b**) of *Pilocarpus microphyllus*, with different initial medium pHs, during 30 days of cultivation

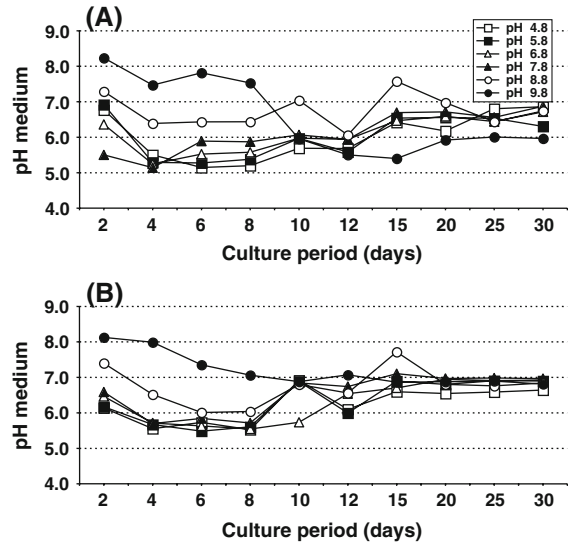

Fig. 2 pH variation in the culture medium of lineage P (**a**) and PP (**b**) of *P. microphyllus*, with different initial medium pHs, during 30 days of cultivation

Nitrate content decreased in the media during cell culturing (Fig. 3a, b). The content in lineage PP was lower than lineage P on the 2nd day, but remained higher on the 12th day. The content of NH_4^+ varied very similarly in both lineages at the various pHs (Fig. 3c, d). It is noteworthy that for both lineages at pH 9.8, the NH_4^+ content was very low beginning on the 2nd day.

Lineage P and PP produced significant amounts of pilocarpine, while lineage PP produced pilosine as well. Alkaloid production was always higher in the cells. These results are presented separately in the following sections.

Lineage P

Cells of this lineage produced and released pilocarpine into the medium. Unexpectedly, the highest pilocarpine production occurred at high pHs, mainly after the 20th day. At pHs 8.8 and 9.8 (Fig. 4a), the cells were still viable after 30 days, as shown by viability testing carried out with fluorescein diacetate (Balestri and Cinelli 2001). At all pHs, the pilocarpine concentration in the cells was strongly reduced on the 30th day (Fig. 4a). Pilocarpine was also highest at high media pHs and at all pHs there was a marked decrease of alkaloid concentration on the 30th day (Fig. 4b).

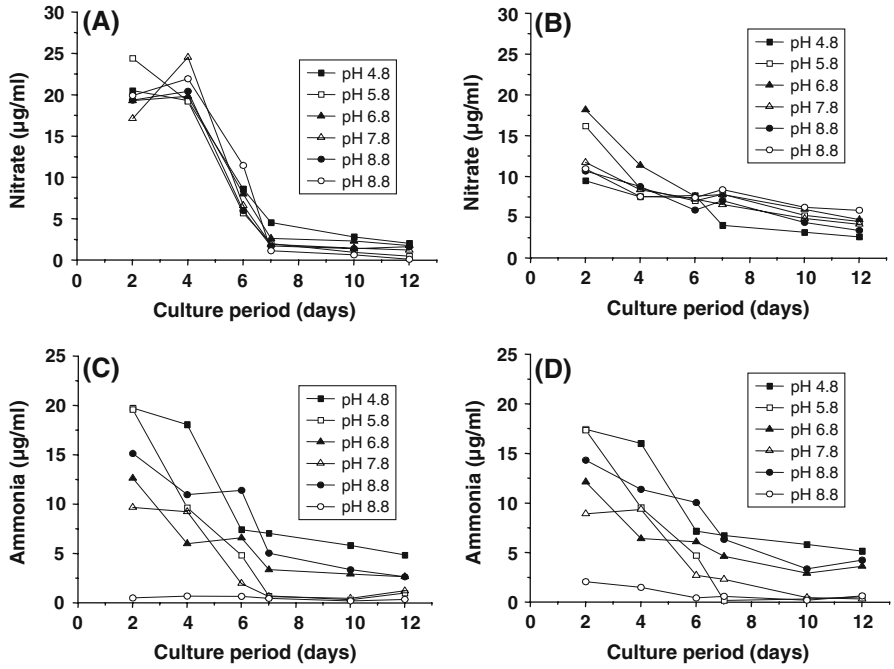

Fig. 3 Nitrate (**a**, **b**) and ammonium (**c**, **d**) contents in the culture media of lineages P (**a**, **c**) and PP (**b**, **d**) of *P. microphyllus*, with different initial medium pHs, during 12 days of cultivation

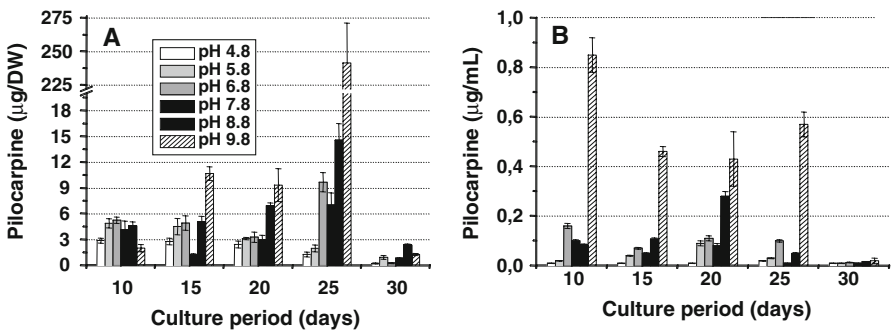

Fig. 4 Pilocarpine contents in the cells (**a**) and in the medium (**b**) of cell culture lineage P (*P. microphyllus*), with different initial medium pHs, during 30 days of cultivation

Lineage PP

In general, the quantities of pilocarpine in the cells and media of lineage PP were higher than the values found in lineage P (Fig. 5a, b). The highest production of pilocarpine in lineage PP occurred after the 20th day, at high pHs (Fig. 5a). At low pHs there was an increase of pilocarpine production during the cultivation period, a phenomenon that was not observed with lineage P. There was also a marked decrease of pilocarpine on the 30th day at all pH treatments. Pilocarpine also accumulated in the medium at high pHs and a decrease of content occurred on the 25th day, 5 days before the decrease observed in the cells (Fig. 5b). Much less of the pilosine alkaloid was produced by lineage PP (Fig. 5c). Except 25th day/pH 5.8, pilosine accumulated in the cells at high concentrations on the 10th or 15th days (Fig. 5c). On the 30th day, there was a marked decrease of pilosine in the cells. In the media, the greatest accumulation of pilosine was always on the 25th day, being highest at pH 9.8 (Fig. 5d). On the 30th day, the pilosine content in the media was also reduced.

In addition to the lack of pilosine in lineage P, there were other variations in the composition of other alkaloids. Mass spectrometry analysis allowed the identification of 6 alkaloids in cells of lineage P and PP, and in the media culture: pilocarpine (*m/z* 209), pilosine (*m/z* 287), anhydropilosine (*m/z* 269), 13-nor-8(11)-dihydropilocarpine (*m/z* 193), 3-hydroxymethyl-

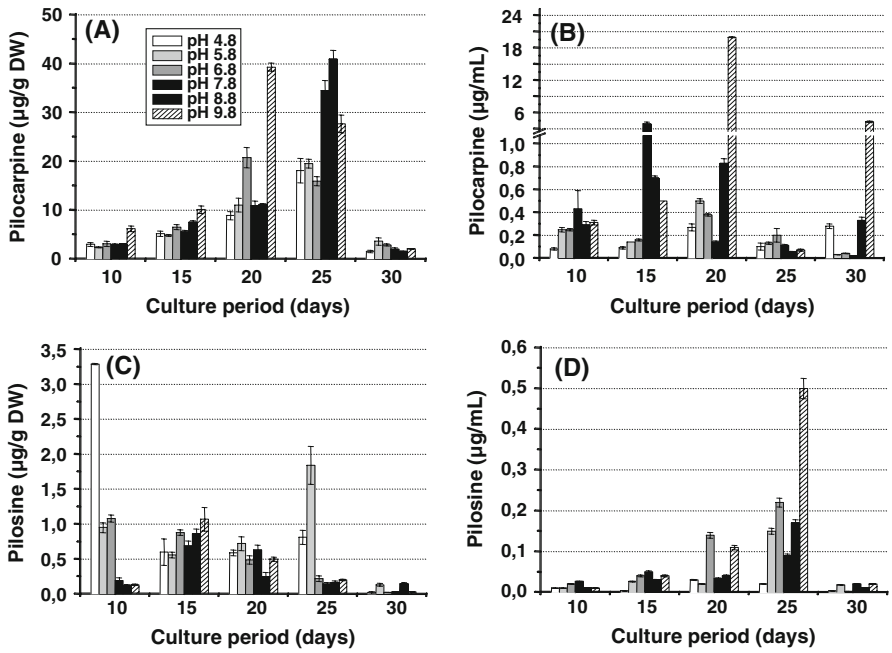

Fig. 5 Pilocarpine (**a**, **b**) and pilosine (**c**, **d**) contents in the cells (**a**, **c**) and in the medium (**b**, **d**) of lineage PP (*P. microphyllus*), with different initial medium pHs, during 30 days of cultivation

4-(3-methyl-3H-imidazol-4-yl)-1-phenyl-butan-1-one (*m/z* 259) and 3-benzoyl-4-(3-methyl-3H-imidazol-4-ylmethyl)-dihydro-furan-2-one (*m/z* 285) (Table 1).

Discussion

The cell suspensions of jaborandi were established from callus cultures produced from petioles from the same *P. microphyllus* plant. Nevertheless, lineages P and PP presented very distinct morphological and physiological characteristics, in terms of cluster formation, growth rate, and alkaloid production. Such variations have been reported in cell suspension cultures of *Catharanthus roseus* (El-Sayed et al. 2004) and *Ocimum basilicum* (Kintzios et al. 2003).

Cell organization may be necessary for the production of some secondary metabolites (Brodelius et al. 1979; Haldimann and Brodelius 1987; Charlet et al. 2000), however, histological preparations made with typical clusters of each jaborandi lineage had no perceptible differences in terms of cell organization. Therefore, it seems that differential alkaloid production in jaborandi cells is not related to cell differentiation.

Pilocarpine which is sold throughout the world comes exclusively from jaborandi plants grown in South America, particularly from the region of the Brazilian Amazon forest (Pinheiro 1997) and, until recently, the leaves used for extraction were collected from native plants growing in the wild, resulting in the addition of jaborandi to the list of Brazilian endangered species.

The industrial extraction of pilocarpine from the jaborandi leaves uses organic solvents. Since pilosine has no commercial use, it is considered a contaminant, and additional steps are necessary for its separation from pilocarpine (Centroflora Group, personal communication). Therefore, lineage P would be interesting for commercial exploration using bioreactors, since it is friable and produces and excretes exclusively pilocarpine. In contrast, although lineage PP produces pilosine and pilocarpine, it is an interesting model to study the biosynthesis of imidazole alkaloids in jaborandi since the biosynthesis routes of pilocarpine and pilosine seem to be related (Abreu et al. 2007b; Sawaya et al. 2008).

Growth of the cell suspension cultures of jaborandi showed highest mass accumulation at high pHs. However, there was no relationship between cell proliferation and alkaloid production in the cells or released in the medium, nor with the total alkaloid production per flask. The lack of a correlational between growth and alkaloid production was also observed in *Camptotheca acuminate,* where the increase of camptothecine occurred in the stationary phase of the cell culture, while the increase of

Table 1 Alkaloid composition in cells and media of cell suspension cultures of *P. microphyllus* at different initial medium pHs on the 30th day of cultivation

Alkaloid	Cell/ medium	Media pH					
		4.8	5.8	6.8	7.8	8.8	9.8
m/z 209 (pilocarpine)	Cell P	x	x	x	x	x	x
	Medium P	x	x	x	x	x	x
	Cell PP	x	x	x	x	x	x
	Medium PP	x	x	x	x	x	x
m/z 287 (pilosine)	Cell P						
	Medium P						
	Cell PP	x	x	x	x	x	x
	Medium PP	x	x	x	x	x	x
m/z 269	Cell P		x	x	x	x	x
	Medium P		x	x	x	x	x
	Cell PP	x	x	x	x	x	x
	Medium PP	x	x	x	x	x	x
m/z 193	Cell P		x	x			
	Medium P					x	
	Cell PP		x	x		x	
	Medium PP		x				
m/z 241	Cell P					x	
	Medium P						
	Cell PP						
	Medium PP						
m/z 259	Cell P		x	x		x	x
	Medium P		x	x		x	x
	Cell PP		x	x	x	x	x
	Medium PP						
m/z 285	Cell P		x				
	Medium P						
	Cell PP		x	x	x	x	
	Medium PP		x	x	x	x	

m/z 269 = anhydropilosine; *m/z* 193 = 13-nor-8(11)-dihydropilocarpine; *m/z* 241 = 3-(3-methyl-3H-imidazol-4-ylmethyl)-1-phenyl-but-3-en-1-one; *m/z* 259 = 3-hydroxymethyl-4-(3-methyl-3H-imidazol-4-yl)-1-phenyl-butan-1-one; *m/z* 285 = 3-benzoyl-4-(3-methyl-3H-imidazol-4-ylmethyl)-dihydro-furan-2-one

isocamptothecines A and B occurred when growth was highest (Van Hengel et al. 1992; Yu et al. 2005). Nevertheless, the data in the literature eludes consensus, since caffeine accumulation occurred in coffee cell cultures during the senescing phase (Buckland and Townsley 1975; Baumann and Rohrig 1989).

Here, the concentrations of the imidazole alkaloids were always higher in the cells and, in general, the greatest accumulation of pilocarpine in the medium and cells occurred on the same day (but not for pilosine in lineage PP). The high production on a specific day and the release of alkaloids to the medium might be an attempt of the cells to buffer the pH of the medium. In aqueous solution, pilocarpine may epimerize to isopilocarpine which, in turn, hydrolyzes to isopilocarpic acid (Abreu et al. 2007b). Unfortunately, diastereoisomers cannot be distinguished from their original structures by the ESI-MS method used here (Abreu et al. 2007a; Sawaya et al. 2008). Together with the other alkaloids detected in the cell and in the media, the pH might be controlled by alkaloid production, release and/or modification of the acidic form.

Curiously, while the pH of all treatments stabilized around 6 after the 15th day in both lineages, the highest pilocarpine production was observed on the 25th day, particularly at pHs 8.8 and 9.8. Therefore, it seems that the medium pH did not affect the production of this alkaloid at the beginning of cell culture, but in some way, there was a long-term effect. It is difficult to consider that pHs 8.8 and 9.8 caused a long-term stress since cells of these treatments grew well until the end of the experiment. Regarding pilosine, the opposite seems to happen, as the highest production of this alkaloid was observed at pH 4.8 and on the 10th day.

Stabilization of the pH in media cultures with initially high pHs has been reported (Takayama et al. 1977; Minocha 1987) and a common explanation for such variations has been the changes in the NH_4^+ and NO_3^- content due to a differential absorption of these nutrients by the cells (Minocha 1987; Salisbury and Ross 1992).

In both lineages the NH_4^+ content was already low on the 2nd day at pHs 6.8–8.8. On the same day, the NH_4^+ content of cells of pH 9.8 flasks was even lower, indicating an intense absorption. However, the medium pH stabilized only at the 10th day for lineage P and at the 15th day for lineage PP. This lack of a relationship between NH_4^+ content and pH stabilization might result from slow absorption of NO_3^-, with a consequent lower release of H^+ into the medium.

Pilocarpine production per flask (cells + culture medium) varied during the experiment. Therefore, either the alkaloids were degraded (intra- and extracellularly) or, after release, they were re-absorbed and degraded inside the cells. Some plant cell suspension

cultures can absorb alkaloids previously released into the medium (Sakai et al. 2002; Terasaka et al. 2003; Goodman et al. 2004) but the control of such re-absorption and the transport of this class of compounds through membranes is still largely unexplored.

Two mechanisms are known for the transport of secondary metabolites across membranes of plant cells: a system involving a H^+ gradient, mediated by a H^+-antiport, and a second system called the ATP-Binding Cassette (ABC) (Martinoia et al. 2002; Yazaki 2006). Control by protein membrane transporters might explain the variation in pilocarpine and pilosine levels in the cells and in the culture medium in all pH treatments. To our knowledge, no study had thus far focused on the effect of medium pH as opposed to membrane transporters in the control of plant cell secondary metabolite content. Pilosine accumulated in the cells in acidic medium in the first days of the experiments, while the opposite was observed for pilocarpine, which accumulated when the pH had stabilized to around 6.

Therefore, an important next step it is to investigate the proteins mediating the transport of pilocarpine and pilosine in the jaborandi cells, using specific inhibitors. Further research is necessary to determine how the effect of medium pH can change the response of these proteins, in order to increase the release of alkaloids to the medium as a way of making their economical exploitation possible using bioreactors.

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