J. Microbiol. Biotechnol. (2013), **23**(5), 656–660
http://dx.doi.org/10.4014/jmb.1211.11035
First published online March 12, 2013
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Nutritional Studies on Production of Antibacterial Activity by the Zebra Mussel Antagonist, *Pseudomonas fluorescens* CL0145A

Polanski-Cordovano, Grace, Lea Romano, Lauren L. C. Marotta, Serena Jacob, Jennifer Soo Hoo, Elena Tartaglia, Deepa Asokan, Simkie Kar, and Arnold L. Demain*

Research Institute for Scientists Emeriti (R.I.S.E.), Drew University, Madison, NJ 07940, USA

Received: November 15, 2012 / Revised: January 4, 2013 / Accepted: January 7, 2013

Pseudomonas fluorescens **strain CL0145A was discovered at the New York State Museum Field Research Laboratory as an effective agent against the environmentally destructive zebra mussel, which has contaminated US waters. Dried cells of the microbe are being commercialized as an environmentally friendly solution to the problem. We found that antibiotic activity against the Gram-positive bacterium *Bacillus subtilis* is produced and excreted by this strain. We have carried out studies to optimize production of the antibiotic. Studies were begun in a complex corn meal medium. Activity was found in both cells and culture supernates and was maximal after one day of fermentation. Static fermentation conditions were found to be superior to shaken culture. Production of extracellular antibiotic in complex medium was found to be dependent on the content of sucrose and enzyme-hydrolyzed casein. Indeed, production was greater in sucrose plus enzyme-hydrolyzed casein than in the complex medium. Of a large number of carbon sources studied as improvements over sucrose, the best was glycerol. An examination of nitrogen sources showed that production was improved by replacement of enzyme-hydrolyzed casein with soy hydrolysates. Production in the simple glycerol-Hy-Soy medium was not improved by addition of an inorganic salt mixture or by complex nitrogen sources, with the exception of malt extract. In an attempt to keep the medium more defined, we studied the effect of amino acids and vitamins as replacements for malt extract. Of 21 amino acids and 7 vitamins, we found tryptophan, glutamine, biotin, and riboflavin to be stimulatory. The final medium contained glycerol, Hy-Soy, tryptophan, glutamine, biotin, and riboflavin.**

*Corresponding author
Phone: +1-973-4083937; Fax: +1-973-4083504;
E-mail: ademain@drew.edu

Key words: *Pseudomonas fluorescens*, microbial nutrition, antibacterial, zebra mussels

Zebra mussels (*Dreissena polymorpha*) are major environmental problems in North American freshwaters. These small freshwater bivalves have spread throughout these waters since they were unintentionally introduced from Europe in the 1980s. Adverse economic and ecological problems have developed since they colonize raw water-dependent infrastructures, such as water intake structures on boat motors, pipes in electrical power infrastructures, *etc*. They clear rivers and lakes of vital plant life and also infect and kill larger bivalves. They cause damage and lead to increases in operating expenses amounting to hundreds of millions of dollars. The environmental damage includes a decrease in phytoplankton productivity, an increase in native bivalve mortality, and restructured benthic communities. Measures to control zebra mussels have been explored, such as thermal and chemical treatments of water, but these have undesirable environmental effects. It was clear that new biocontrol agents were necessary. By screening hundreds of microbes, researchers of the New York State Museum laboratory found that *Pseudomonas fluorescens* strain CL0145A (ATCC 55799) could selectively kill zebra mussels when ingested by these filter-feeders. The strain originated from soil in a North American river and is the first biological control agent for zebra mussels [9, 10]. The effectiveness of the bacterium has been observed in laboratory and field trials [11]. The death of the mussels has been shown to be *via* a toxin as opposed to infection by the bacterium [6]. Research at the NY State Museum has shown that the cell-associated toxin is produced as a heat-labile (50°C for 30 min) compound that is denatured at higher temperatures [12]. Scale-up of the *P. fluorescens*

strain was accomplished at the 100 liter level [7]. Dried cells of *P. fluorescens* CL0145A are now being commercialized as Zequanox by Marrone Bio Innovations [8].

We have found that *P. fluorescens* strain CL0145A produces and excretes antibiotic activity against *Bacillus subtilis*. This may or may not be related to the zebra mussel toxin. Antibacterial activity can be demonstrated and measured in a simple overnight agar diffusion assay. Antibiotic production was found to be rapid, appearing after only one day of fermentation. Culturing in a complex corn meal medium (CMM) led to production of the antibiotic and excretion into the extracellular broth. The present work describes development of a more defined medium including the effect of carbon and nitrogen sources on production of the antibiotic.

Materials and Methods

Producing Organism

P. fluorescens CL0145A was obtained from the New York State Museum, New York State Education Department, Albany, NY, USA. It was stored in 30% glycerol in the freezer at −75°C.

P. fluorescens Seed Preparation

The seed medium was BBL Trypticase Soy Broth (TSB) used at 25 ml in a 250 ml unbaffled Erlenmeyer flask. The flask was inoculated with 0.5 ml of a *P. fluorescens* CL0145A stock culture. The seed was grown for 22 h at 26°C on the shaker at 200 rpm.

P. fluorescens Fermentation

The initial fermentation medium was the complex corn meal medium (CMM) [2]. It contains 25 g of sucrose, 25 ml of molasses (Brer Rabbit), 2.5 g of Sigma corn steep solids, 10 g of Bacto-malt extract, 10 g of Sigma casein enzymatic hydrolysate (EHC), and 2 g of K_2HPO_4 in 1 L of distilled water. The pH was adjusted to 7.0 before autoclaving. Duplicate flasks (250 ml) containing 100 ml of CMM were inoculated with 0.4 ml of seed culture and either incubated at 26°C without agitation or on the shaker at 200 rpm. Samples were taken for assay at 1 and 2 days, centrifuged at 4°C and 5,000 rpm to remove cells, and the supernatants were assayed by the *B. subtilis* agar diffusion assay.

B. subtilis Assay

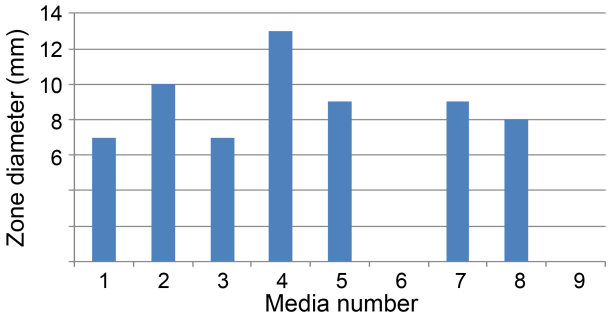
B. subtilis strain 168-1 (ATCC 27370) seed was prepared in a flask of TSB inoculated with 75 µl of *B. subtilis* stock culture. It was incubated on the shaker at 200 rpm at 26°C for 72 h. Then 1.2 ml of the seed was inoculated into a 250 ml flask containing 200 ml of Luria–Bertani (LB) agar at approximately 55°C, and distributed into Petri dishes at 9 ml per dish. The plates were refrigerated until used. To assay *P. fluorescens* fermentation broths or their supernatants, 0.5 ml was aseptically transferred from the flask into test tubes. Duplicate paper disks (6 mm diameter) were dipped into the broths, dried on aluminum foil, and placed on the *B. subtilis* plates. The plates were incubated overnight at 37°C and the diameters of clear zones were measured.

Results

Initial Experiments on the Production of Antibacterial Activity in Complex and Simpler Media

Our initial objective was to develop a medium that is less complex than CMM. Initial studies compared production in complex media CMM [2], Tryptic Soy Broth (TSB) [9], and nutrient broth. It was found that production occurred in all three media but was somewhat higher in CMM. An experiment was carried out by eliminating components of CMM (medium 1 of Fig. 1). When malt extract was eliminated from CMM (medium 2), antibiotic production increased somewhat. Further omissions had variable results. The most striking observation was that elimination of enzymatic hydrolysate of casein (EHC) (media 6 and 9) resulted in total loss of antibiotic activity. The data also indicated that the more defined medium 8, containing only EHC, sucrose, and potassium phosphate, was suitable for production, and indeed was more productive than the much more complex CMM. A further experiment was done in a simpler medium containing EHC and sucrose (Table 1), and the data showed the superiority of sucrose-EHC medium over complex CMM and TSB.

Variation in the concentration of sucrose in the sucrose-EHC medium showed that either reducing it by 50% (12.5 g/l) or doubling its concentration (50 g/l) reduced production. The site of antibiotic activity was studied next. Assays were done on the whole broth of the culture, the culture supernatant, and the cell pellet. The cell pellet was



Component	1	2	3	4	5	6	7	8	9
Malt extract	+	-	-	-	-	-	-	-	-
Molasses	+	+	+	-	+	+	+	-	+
Corn Steep	+	+	+	+	-	+	-	-	-
EHC	+	+	+	+	+	-	+	+	-
Sucrose	+	+	-	+	+	+	-	+	+
KH_2PO_4	+	+	+	+	+	+	+	+	+

Fig. 1. Effect of eliminating components of the CMM on antibacterial activity.

Table 1. Antibiotic production in complex media TSB, CMM, and in semi-defined sucrose-EHC medium.

Medium	Inhibition zone diameter (mm)
TSB (Tryptic soy broth)	7
CMM (Corn meal medium)	9
Sucrose-EHC	14

Table 2. Antibiotic activity in whole broth, supernatant, and cell pellet.

Sample	Zone diameter (mm)
Whole broth	21
Cell pellet	18
Supernatant	13

Table 3. Antibiotic production in glycerol-EHC medium.

Fermentation condition	Inhibition zone diameter (mm)	
	1 day	2 days
Static	16	15
Shaken	8	13

Table 4. Effect of concentrations of glycerol and Hy-Soy on antibiotic production.

Glycerol (g/l)	Hy-Soy (g/l)	Zone diameter (mm)
25 (control)	10 (control)	11
12	10	12
48	10	11
25	5	13
25	1	10

suspended in 5 ml of distilled water before assay. Table 2 shows that whole broth contained the greatest amount of antibiotic activity and the cell pellet had greater activity than the supernatant. However, supernatants were used for further experiments since their zone edges upon disk assay were much sharper than those with cell pellets or whole broth, and unlike zones with whole broth or cell pellets, they did not have *Pseudomonas* growth around the discs.

Effects of Carbon Sources

We next compared sucrose with other carbon sources. Carbon sources tested were starch, dextrin, glucose, lactose, fructose, maltose, rhamnose, mannitol, inositol, glycerol, trehalose, raffinose, xylose, galactose, arabinose, and sorbose. All except xylose, galactose, arabinose, and sorbose supported some degree of production. Supporters of a high level of production were glycerol, trehalose, fructose, maltose, lactose, starch, and raffinose. For later experiments, it was decided that glycerol would replace sucrose since it was one of the best carbon sources.

Static vs. Shaken Fermentation

Data confirming production in glycerol-EHC medium and showing the superiority of static culture vs. shaken culture are shown in Table 3. In static culture, zone sizes were higher on day 1 than on day 2, indicating that inactivation of the antibiotic occurred after the peak of production was reached. With the slower production in shaken culture, titers kept increasing up to day 2. Subsequent experiments were done using static cultures.

Effects of Nitrogen Sources

We next examined the effects of different nitrogen sources. We compared EHC with soy hydrolyates (Bacto-Soytone and Quest Hy-Soy), urea, Pharmamedia, corn steep solids, tryptose, and fish meal. It was found that the soy hydrolysates were somewhat more effective than EHC, whereas the others were poorer. Hy-Soy was chosen as the nitrogen source for subsequent experiments.

Concentration of Medium Ingredients

The effect of concentrations of glycerol and Hy-Soy on antibiotic production was examined next. The data in Table 4 show that decreasing the Hy-Soy level to 5 g/l yielded the best production. Increasing or decreasing the level of glycerol had no or minimal effects. Subsequent experiments were done with 25 g/l glycerol plus 5 g/l Hy-Soy.

Time Course of Production

Examination of the time course of production in glycerol-Hy-Soy medium was carried out by assaying at 2 h intervals between 16 and 30 h. It was found that zone size peaked at 24 h and decreased thereafter.

Effects of pH and Additional Complex and Semi-Defined Nitrogen Sources

A study involving the effect of pH adjustment of the glycerol-Hy-Soy medium (from 5.5 to 8.5) on antibiotic formation showed that pH values between 7.0 and 8.0 gave optimum production. As described in Materials and Methods, all experiments up to this point had utilized adjustment to pH 7.0.

The effect of adding additional crude and semi-defined nitrogen sources to the glycerol-Hy-Soy medium showed no further stimulation of antibiotic production occurred; indeed, production was inhibited to various extents (Table 5). A further study utilizing other additional complex nitrogen sources (Table 6) revealed slight stimulation by malt extract.

Effects of Inorganic Salts and Amino Acids

Stimulation by malt extract was confirmed in an additional experiment. Since malt extract is a complex nitrogen

Table 5. Effect of adding complex nitrogen sources to glycerol-Hy-Soy medium on antibiotic production.

Additive (10 g/l)	Zone diameter (mm)
None	19
Bacto-Soytone	17
Casein enzymatic hydrolysate	16
Fish meal	15
Tryptose	13
Yeast extract	9
Corn steep solids	NZ[a]
Fermamine	NZ

[a]NZ = no zone.

source, we were not interested in including it in our test medium. However, we were interested in determining whether inorganic salts or amino acids have positive effects. A mix containing NaCl, $MgS0_4$, $ZnSO_4$, $MnSO_4$, $FeSO_4$, $(NH_4)_2Mo_7O_{24}$, $NaB_4O_7{\cdot}H_2O$, $CoCl_2$, and $CuCl_2$, adjusted to pH 6.5 and tested at four different concentrations, had little to no effect on production. Ammonium phosphate was found to be able to replace Hy-Soy as a growth substrate, to produce a similar pH pattern, but yielded no inhibitory zone on the *B. subtilis* assay plate. We next investigated whether L-amino acids stimulate or inhibit antibiotic formation.

Addition of L-amino acids to the glycerol-Hy-Soy medium employed concentrations of 0.5, 1, and 3 g/l. The amino acids tested were ornithine, tyrosine, proline, valine, tryptophan, methionine, serine, glycine, phenylalanine, arginine, isoleucine, leucine, lysine, aspartic acid, alanine, threonine, cystine, glutamine, asparagine, histidine, and glutamic acid. Although many had no effect, zone size was significantly increased by 15–25% *via* addition of tyrosine, valine, glutamine, glutamic acid, asparagine, or tryptophan. On the other hand, cystine totally inhibited antibiotic production. When various combinations of the stimulatory amino acids were tested, it was found that the stimulation by a mix of tryptophan and glutamine (22%) was not increased by addition of the remaining stimulatory amino acids. The time course of fermentation in the medium containing 25 g/l glycerol, 5 g/l Hy-Soy, 0.5 g/l tryptophan, and 0.5 g/l glutamine was studied by assaying every hour from 12 to 32 h. Peak activity was observed at 26 h followed by decreasing zone sizes.

Table 6. Effects of adding complex nitrogen sources to glycerol-Hy-Soy medium on antibiotic production.

Additive (10 g/l)	Zone diameter (mm)
None	13
Malt extract	14
Beef extract	13
Soy grits	11
Gluten enzymatic hydrolysate	9
Pharmamedia	NZ

Table 7. Effects of adding vitamins on antibiotic production.

Additive (mg/l)	Zone diameter (mm)
None	17
Biotin (1)	18
Biotin (10)	19
Biotin (50)	19
Riboflavin (1)	18
Riboflavin (10)	19
Riboflavin (50)	18

Effects of Vitamins

Effects of vitamin addition were studied in the glycerol, Hy-Soy, tryptophan, and glutamine medium. The vitamins were tested at 1, 10, and 50 mg/l. Those that did not stimulate production were thiamine, vitamin B_{12}, L-ascorbic acid, pyridoxine, and nicotinic acid. Stimulatory vitamins were biotin and riboflavin (Table 7).

Future studies to identify the antibiotic(s) produced will employ the final medium developed here, containing 25 g/l glycerol, 5 g/l Hy-Soy, 0.5 g/l tryptophan, 0.5 g/l glutamine, 10 mg/l biotin, and 10 mg/l riboflavin.

Production of Antibiotic by a Mutant Incapable of Producing Hydrogen Cyanide (HCN)

One concern we had during this work was that the antibacterial agent might be HCN, which is known to be produced by species of *Pseudomonas*. To examine this possibility, we tested production by an HCN-negative mutant of *P. fluorescens* CL0145A. However, it was able to produce the antibiotic, thus showing that our inhibitory activity was not due to HCN.

Discussion

It is not surprising that *P. fluorescens* CL0145A produces an antibiotic. Many strains of this species produce such compounds [1, 3, 4, 14–16]. *P. fluorescens* NCIMB 10586 produces mupirocin, which is used as a topical treatment for staphylococcal infections [3]. Other strains produce 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and *N*-mercapto-4-formylcarbostyril [4]. Strain *P. fluorescens* MSS-1 is lethal to three mosquito species larvae owing to a toxin [13]. Many strains of *P. fluorescens* effectively protect plants such as wheat and cotton against plant diseases by producing antibiotics [5]. Our work has shown that strain CL0145A produces an excreted antibiotic, which may or may not be identical to the zebra mussel-destroying agent.

We have developed a medium for its production containing glycerol, Hy-Soy, tryptophan, glutamine, biotin, and riboflavin, which should be useful for further work to identify the antibacterial compound(s) produced. Marrone Bio Innovations is commercializing the *P. fluorescens* CL0145A cell system and will produce it in Bangor, Michigan [8].

Acknowledgments

We thank Drs. Daniel Malloy and Denise A. Mayer of the New York State Museum for providing the producing culture *P. fluorescens* CL0145A. We are also indebted to Dr. Mayer for providing the HCN-negative mutant. We thank Dr. Pamela G. Marrone, CEO and Founder of Marrone Bio Innovations, Inc., as well as Dr. Raymond Lam, formerly of Marrone Bio Innovations, for their cooperation. Support for the project was provided by the Drew Summer Science Institute (D.S.S.I.) of Drew University. The authors are pleased to acknowledge the Fellows of the Research Institute for Scientists Emeriti (R.I.S.E.), its former director (now deceased) Ashley Carter, the present Director, Jon Kettenring, and Administrator Miriam Donohue for their encouragement of this research. We thank Maria Falzone and Vincent Gullo for assistance in preparing the manuscript. All the authors of this paper except A. Demain and Jennifer Soo-Hoo were undergraduate students of Drew University. Jennifer SooHoo was a high school student.

REFERENCES

1. Abbas, A., J. P. Morrissey, P. C. Marquez, M. M. Sheehan, I. R. Delany, and F. O'Gara. 2002. Characterization of interactions between the transcriptional repressor PhlF and its binding site at the *phlA* promoter in *Pseudomonas fluorescens* F113. *J. Bacteriol.* **184:** 3008–3016.
2. Elander, R. P., J. A. Mabe, R. L. Hamill, and M. Gorman. 1971. Biosynthesis of pyrrolnitrins by analogue-resistant mutants of *Pseudomonas fluorescens*. *Folia Microbiol*. **16:** 156–165.
3. El-Sayed, A. K., J. Hothersall, and C. M. Thomas. 2001. Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB 10586. *Microbiology* **147:** 2127–2139.
4. Fakhouri, W., F. Walker, V. Bernhard, W. Armbruster, and H. Buchenauer. 2001. Isolation and identification of *N*-mercapto-4-formylcarbostyril, an antibiotic produced by *Pseudomonas fluorescens. Phytochemistry* **58:** 1297–1303.
5. Fiechter, A. 2000. *History of Modern Biotechnology 1*. Springer-Verlag, Berlin.
6. Mayer, D. A., D. P. Molloy, M. J. Gaylo, and K. T. Presti. 2003. Small-scale flow-through application of *Pseudomonas fluorescens* strain CL0145A in the service water of a hydropower facility for the biological control of zebra mussels. Abstract P106. Programs and Abstracts of the Annual Meeting of the Society for Industrial Microbiology, Minneapolis.
7. Mayer, D. A., D. P. Molloy, J. T. Morse, K. T. Presti, P. M. Sawyko, and P. A. Sprague. 2004. Biocontrol of zebra mussels: The path to commercialization of *Pseudomonas fluorescens* strain CL0145A. Poster P1 at the Annual Meeting of the Society for Industrial Microbiology, Anaheim
8. McCoy, M. 2012. Marrone constructing biopesticide plant. *Chem. Eng. News* **90:** 16.
9. Molloy, D. 2001. Method for controlling *Dreissena* species. US Patent 6,194,194.
10. Molloy, D. P. 1998. The potential for using biological control technologies in the management of *Dreissena* spp. *J. Shellfish Res.* **17:** 177–183.
11. Molloy, D. P. 2001. Technical Report: Assessing the impact of continuous bacterial treatments over a two-week period on zebra mussel mortality. Available at http://www.osti.gov/servlets/pur1/784620-A92ajd/native/784620.pdf (Feb. 2002).
12. Molloy, D. P., D. A. Mayer, M. J. Gaylo, K. T. Presti, A. Y. Karatayev, and L. E. Burlakova. 2002. Biological control of zebra mussels with microbial toxin: An overview of research progress. Poster, Division of Research and Collections, New York State Museum.
13. Murty, M. G., G. Srinivas, and V. Sekar. 1994. Production of a mosquitocidal exotoxin by a *Pseudomonas fluorescens* strain. *J. Invert. Pathol.* **64:** 68–70.
14. Nakata, K., A. Yoshimoto, and Y. Yamada. 1999. Promotion of antibiotic production by high ethanol, high NaCl concentration, or heat shock in *Pseudomonas fluorescens* S272. *Biosci. Biotechnol. Biochem*. **63:** 293–297.
15. Natch, A., C. Keel, N. Hebecker, E. Laassik, and G. Defago. 1997. Influence of biocontrol strain *Pseudomonas fluorescens* CHAO and its antibiotic overproducing derivative on the diversity of resident root colonizing pseudomonads. *FEMS Microbiol. Ecol.* **23:** 341–352.
16. Sarniguet, A., J. Kraus, M. D. Henkels, A. M. Muehlchen, and J. E. Loper. 1995. The sigma factor σ^s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proc. Natl. Acad. Sci. USA* **92:** 12255–12259.