

Reaching Fish Farms Through AquaFish CRSP Technology Transfer: Elimination of MT from Intensive Masculinization Systems Using Bacterial Degradation

Mitigating Negative Environmental Impacts/Study/09MNE07UA

Wilfrido M. Contreras-Sánchez, Mario Fernández-Perez, Carlos A. Alvarez-González, Arlette Hernández Franyutti, Ulises Hernández-Vidal, Alejandro Mcdonal-Vera
Universidad Juárez Autónoma de Tabasco
Villahermosa, Tabasco, Mexico

Kevin Fitzsimmons
University of Arizona
Tucson, Arizona

OBJECTIVES

The general objective of this project is to transfer A&F CRSP-generated technology to the private sector, ensuring the development of sustainable tilapia aquaculture in Southeastern Mexico and Central America. The specific objectives are:

1. Evaluate combination of bacteria that best degrade MT in tilapia masculinization systems.
2. Transfer MT degrading technology using bacteria to a large hatchery.
3. Develop written materials for the dissemination of these protocols and train tilapia farmers in the use bacterial bio-flocks as MT-degrading entities and probiotics.
4. Continue evaluation of bacterial combinations that improve degradation of MT and that can serve as probiotics

INTRODUCTION

Aquaculture industry considers different technologies in order to improve food production for the increasing human needs. One alternative for male monosexual population production is the utilization of synthetic steroids. The aquaculture industry commonly uses steroids including the 17α – methyltestosterone (MT), miblerolone, fluoxymesterone, nortestosterone acetate, 17α – methylidihydrotestosterone and norgestrel. MT is the most common form in worldwide use (Hurtado, 2005; López et al., 2007). Specifically, MT is a strong androgen widely used for tilapia masculinization. Around 90% of MT used during masculinization treatments is degraded during the first 24 h and only 1% is remaining 3 weeks later in the fish body (Raisz et al., 2006; Contreras, 2001).

Other natural and synthetic chemicals present in the environment like 17α - estradiol, estriol, estrone, progesterone, testosterone and phytoestrogens mimic hormonal actions. These chemicals actually are considered a worldwide issue because they could disturb sexual characteristics in fish, amphibians, reptiles and mammals (Toft et al., 2004; Argemi et al., 2005; Bracho, 2007). These compounds are considered Endocrine Disrupting Chemicals (or Compounds) or EDCs because are exogenous chemicals which could promote endocrine system alterations and adverse effects on individuals, broods and populations health (Anonymous, 2002). This phenomenon includes reproductive system dysfunction, neoplasia, malformations, neurotoxicity, and decreasing immune response, promoting difficulties to detect possible causes and effects (Swan et al., 2003; Rivas et al., 2004).

In order to decrease environmental impacts of these compounds, Best Management Practices for aquaculture include the use of Recirculation Aquaculture Systems, (RAS). RAS decreases the total waste production, saving water and decreasing waste in the fish culture systems improving water quality for reutilization. Another method for chemical elimination of hormones is the use of granular activated charcoal to absorb the compounds. Finally, phytosteroids have been tested from vegetal

origin; these compounds mimic fish steroids and sometimes have similar impacts on gonadal development (Contreras, 2001; Campos, 2004).

Microbiological degradation processes have been widely studied in recent years; microorganisms have biodegrading features for many pollutants. Microorganisms accumulate, transform or degrade different pollutants including petroleum hydrocarbons. In order to decrease risks for human and environment health the main scope for this study was to determine bacterial strains able to degrade 17 α – methyltestosterone (MT). These bacteria were isolated from a biofilm obtained from a RAS used for fish chemical sex reversion.

MATERIALS AND METHODS

Bacterial strains selection

Strains were obtained from the Microbiology Laboratory at the División Académica de Ciencias Biológicas at UJAT. These strains were originally isolated from biofilms obtained from biological filters used in a tilapia (*Oreochromis niloticus*) masculinization system. Strains of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Bacillus subtilis* were selected according to previous experimental data showing potential to degrade MT under culture conditions where these steroids were used as carbon and energy source.

Bacterial strains adaptation

The four bacteria strains were subjected to adaptation to MT as main source of carbon and energy during 10 days-period, previous to experimental procedure. Each bacterial strain was inoculated in a glass beaker containing 100 mL of liquid mineral culture media containing 40 mg of MT (Sigma-Aldrich, Inc.) according to the procedure presented by Pérez *et al.*, (2006), Yamanaka *et al.*, (2007) and Montpas *et al.*, (1997) respectively (Table 1). All treatments were evaluated in triplicate, including control treatments containing MT and no bacteria, and without MT and bacteria present. Cultures were incubated at 30 ± 1 °C in an orbital shaker incubator 175 rpm, (PolyScience®) (Perez *et al.*, 2006; Yamanaka *et al.*, 2007).

During the experimental phase, the number of viable cells was estimated by conventional plate counting technique. MT-enriched liquid mineral medium was used adding 15 g per liter of bacteriological agar (BIOXON®). Each sample was serially diluted (1:10) in a sterile 0.85% saline solution to obtain a 10^{-5} final dilution. For each diluted sample, 100 μ l were inoculated in the center of each Petri dish and extended using a sterile Drigalski spreader. Inverted Petri plates were incubated for 48 h to 30 ± 0.5 °C and the count were estimated using a Darkfield Quebec colony counter (Bracho *et al.*, 2004).

Table 1.- Chemical composition of the culture media used g/L. (-) indicated the absence of the mineral compound.

Minerals	<i>Bacillus</i>	<i>Pseudomonas</i>	Modified
NH ₄ Cl	-	0.1	0.005
MgSO ₄ · 7H ₂ O	0.1	0.05	0.1
K ₂ HPO ₄ · 3H ₂ O	6.3	0.1	6.3
KH ₂ PO ₄ · 3H ₂ O	1.83	0.05	1.83
FeSO ₄ · 7H ₂ O	0.1	0.001	0.1
CaCl ₂	0.1	0.001	0.1
(NH ₄) ₂ SO ₄	1	-	1
KCl	-	0.01	0.01

Determination of MT to evaluate degradation capacity

The experiment had 4 treatments with controls: 1) *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*; 2) *Bacillus subtilis* and *Bacillus cereus*; 3) *Pseudomonas aeruginosa* and *Bacillus cereus*; and 4) *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Bacillus subtilis* and *Bacillus cereus* mineral culture media and MT, without bacteria; and 2) A flask with mineral culture media without MT and with the corresponding bacteria according to the treatment. Each treatment was performed in triplicate, but the treatment control had only one experimental unit.

Each treatment was inoculated with 1 mL of a bacterial suspension equal to the Turbidimetry McFarland scale of 0,5 and corresponding to a population of $1,5 \times 10^8$ UFC/ 100 mL. The inoculum was incubated at $30^\circ\text{C} \pm 1^\circ\text{C}$ with rotary agitation at 175 rpm (PolyScience®) during 22 days.

The number of microorganisms in each treatment was measured daily through viable counting with the superficial extension technique. The media used in this phase was a mineral media enriched with MT with the addition of 15 g of bacteriological agar (BIOXON®) by liter of media. Serial dilutions were made from 1 mL of sample in 9 mL of sterile saline solution at 0,85% (10⁻¹) up to a dilution of 10⁻⁶. From each dilution 100 µL was inoculated in the center of a Petri dish, extending over the media surface with a sterile Drigalski dipstick. The Petri dishes were placed in an inverted position for incubation at $30^\circ\text{C} \pm 0.5^\circ\text{C}$. The dish readings were performed at 48 h using a Darkfield Quebec counter.

Elisa test or Immunoassay method (r-biopham)

Samples from each treatment were collected in order to determine MT concentrations at 0, 2, 6, 10, 16 and 20 days. All samples were stored in a freezer (-20°C) in Eppendorf tubes until analysis. Samples were diluted in methanol at 40% up to a dilution of 10⁻⁶, in order to determine the maximum and minimum limits of MT in the calibration curve of the kit.

The quantitative measurement of MT was performed by the Elisa test or Immunoassay method (r-biopham): 50 µl of sample of each treatment were inoculated by duplicate. Each analysis had six standards in order to obtain the calibration curve of MT. Once the samples inoculated, 50 µl of the conjugate and 50 µl of the antimetiltosterone (antibody) solution was added. Incubation was performed for 12-16 h at $2-8^\circ\text{C}$. After incubation two distilled water rinses were completed, using a semiautomatic washer.

Following the wash step, 50 µl of substrate and 50 µl of chromogen were pipetted into each well and they were incubated in darkness for 30 minutes. Finally 100 µl of stop solution was added to each well and we proceeded to the reading with a spectrophotometer at an absorbance of 450 nm. The obtained data was analyzed with the software RIDA® SOFT Win in order to obtain the MT concentration in each sample. After obtaining the raw data the number was multiplied by the dilution factor.

RESULTS FOR OBJECTIVES 1 AND 4

The treatment which showed the greatest growth after 24 h and a greater number of cells was *P. aeruginosa* and *P. fluorescens*. With a growth curve of 30 days, the treatment with *B. cereus* and *B. subtilis* showed growth at 48 h with a duration of 22 days. Figure 1 shows the growth curves of the two finalized treatments.

DISCUSSION

Objectives 1 and 4

The results obtained with respect to the two treatments demonstrated bacteria growth in MT media (40 mg/100 ml) as the only carbon source. Yomokito et al. (2004) and Ke et al. (2007), observed bacteria growth and biodegradation from 100 mg/ 100 ml of 17β – estradiol and 17α -methyl estradiol and 3-methoxy- 17β -hydroxy-1,3,5 (10),8(9)-d-4-estren (MHE) respectively. In both studies the strains came from activated sludge from a treatment plant with estrogen residues. Jiang et al. (2010), isolated *B. subtilis* as degrading of 17β – estradiol at a concentration of 1 mg/100 mL which differs with our project. Due to this the strain was isolated in a mineral media with 50 mg/100 mL of

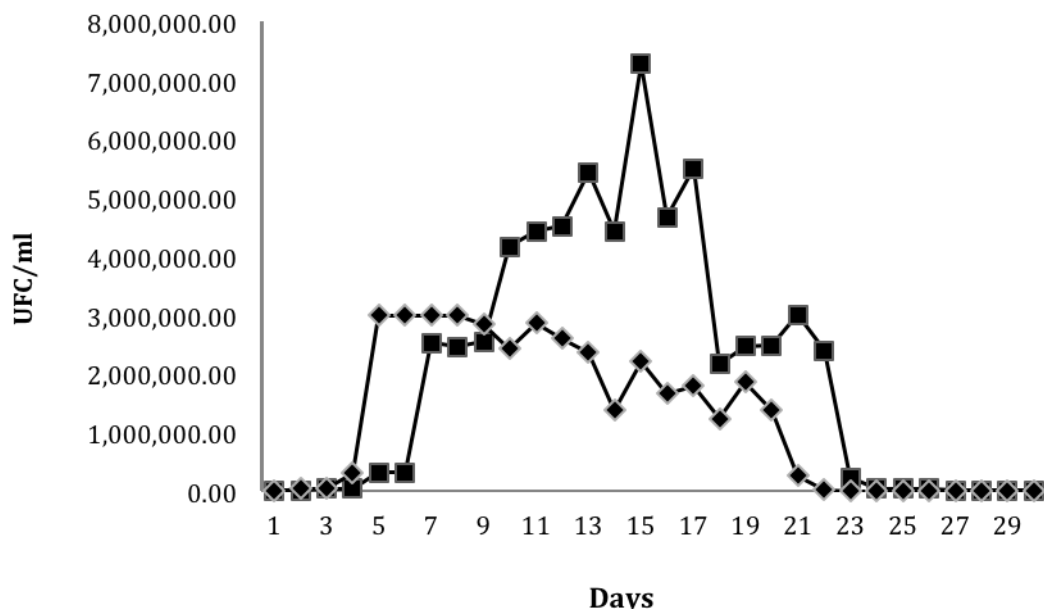


Figure 1. Average value of the microbial growth kinetics in mineral media enriched with 17α -methyltestosterone (MT), the values correspond to mean of triplicate measurements. ■ *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*; ◆ *Bacillus cereus* and *Bacillus subtilis*

estrogen plus 15 mL of activated sludge from sewage of a manufacturer of oral contraceptives in Zhejiang, China, performing successive cycles and changing the concentration up to 1 mg of estrogen.

Steroid biotransformations are important in the manufacture of drugs and hormones; anti-inflammatories, diuretics, steroids, anti-androgenic and anti-cancer agents and with some other applications (Moschet *et al.*, 2009; Walaa *et al.*, 2009). The steroids biotransformation by bacteria and fungi have been suggested by Smith *et al.* (1998) and Walaa *et al.* (2009) investigating the transformation of progesterone by *Apiocrea chrysosperma*, *Botryosphaerica obtusa*, *Bacillus subtilis* and *Aspergillus niger*. Phelps and Popma (2000) mentioned that bacteria and fungi could be responsible of steroids metabolism and they assumed the existence of an androgen microbial degradation in the biofilters of the recirculation systems.

It has been proved that the genera *Nocardia*, *Bacillus subtilis*, *Mycobacterium*, *Bacterium* and *Arthrobacter* act as estrone degrading. Lee and Liu, (2001), Weber *et al.* (2007); Shulan *et al.* (2007) and Jiang *et al.* (2010), have studied the steroids microbial degradation identifying: *Achromobacter xylosoxidans*, *Ralstonia sp.*, *Raoultella thalophilum* and *B. subtilis* as responsible for estrogens biodegradation, although the conditions of our experiment were different, the percentages of biodegradation were between 80 to 85%, which are similar to the above investigations.

The *Pseudomonas sp.* and *Bacillus sp.* have been used in the biodegradation of hydrocarbons, obtaining a good elimination rate, thus it is recommended that both microorganisms can be used for biodegradation of these compounds (Mohamad *et al.*, 2004). Duarte and Gomes (2000) mentioned that degradation increased with a bacteria conjugate due to better degradation capacity with a wider range of action.

Objective 2. Transfer MT degrading technology using bacteria to a large hatchery

Work on this objective is still underway. During the extreme flooding in mid-2011, the hatchery at our demonstration farm partner was lost and the biofiltration system was destroyed. The operation has been moved to a second location that is significantly higher and should be above the flood stage that was breached in each of the last two years. We are collecting data in late 2011 and early 2012.

Objective 3. Develop written materials for the dissemination of these protocols and train tilapia

farmers in the use bacterial bio-flocks as MT-degrading entities and probiotics.

Work on this objective is nearing completion. A biofloc workshop was held in September 2010 at UJAT for hatchery managers who would be working with biofloc systems. Some were interested to use bioflocs to remove residual MT and other more specifically to produce bioflocs as a feed source. A Power Point presentation has been prepared and shared with the farmers.

LITERATURE CITED

- Ahmen H and Naim. Bacterial diversity of tilapia (*Oreochromis niloticus*) cultured in brackish water in Saudi Arabia. *Aquaculture*. 255:266-272
- Argemi F, Cianni N, and Porta A. 2005. Disrupción endocrina: perspectivas ambientales y salud pública. *Toxicología Ambiental*. 39 (3): 291-300.
- Bracho M, Díaz L and Soto LM. 2004. Degradación de Hidrocarburos Aromáticos por Bacterias Aisladas de Suelos Contaminados con Petróleo en el Estado Zulia. *Boletín del Centro de Investigaciones Biológicas*. 38(3): 1-20.
- Bracho RD. 2007. Degradación de p-Nitrofenol por bacterias en los ríos Añasco y Guanajibo y la quebrada de Oro (Puerto Rico). Tesis para optar el grado de Maestro en Ciencia en Biología. Universidad de Puerto Rico. 73 p.
- Campos CB. 2004. Evaluación de sistema de filtración continua con carbón activado para la eliminación de la 17- α metiltestosterona de sistemas intensivos de inversión sexual de *Oreochromis niloticus*. Tesis de maestría. Universidad Juárez Autónoma de Tabasco. Villahermosa, Tabasco. 105 p
- Contreras SWM. 2001. Sex determination in Nile tilapia, *Oreochromis niloticus*: Gene expression masculinization methods, and environmental effects. Thesis for the degree of doctor. Oregon State University, E. U. 193 p.
- Hurtado N. 2005. Inversión Sexual en Tilapias; revisión bibliográfica. Ingenieros consultores. Lima Perú. 42 p.
- Jiang L, Yang J and Chen Jianmeng. 2010. Isolation and characteristics of 17 β – estradiol degrading *Bacillus* spp. strains from activated sludge. *Biodegradation*. 21: 729-736.
- Ke J, Zhuang W, Yew Hoong K, Reinhard M, Tok TL and Hwa TJ. 2007. Characterization of estrogen-degrading bacteria isolated from an artificial sandy aquifer with ultrafiltered secondary effluent as the medium. *Appl Microbiol Biotechnol*. 75:1163–1171
- Leal Y, Reyes M, Álvarez J, Obregón J and Viña X. 2009. Enteropatogenicidad de bacterias aisladas de peces del agua y plancton de su entorno en Venezuela. *Revista Científica*. 19 (5):446-454.
- López CA, Carvajal DL, Botero MC and Phd. 2007. Masculinización de tilapia roja (*Oreochromis spp*) por inmersión utilizando 17 alfa–metiltestosterona. *Revista Colombiana Pecuaria*. 20: 318-326.¶
- Mohan S, Maruthamuthu, Maruthamuthu N, Rajesekar A and Palaniswamy N. Biodegradation of palmarosa oil (green oil) by *Serratia marcescens*. *Int. J. Environ. Sci. Tech*. 4 (2): 279-283.
- Duarte CC and Gomes FS. 2000. Gasoline Biodegradation in different soil microcosms. *Brazilian Journal of microbiology*. 31: 45-49.
- Montpas S, Samson J, Langlois E, Lei J, Pichè Y and Chenevert R. 1997. Degradation of 2,4,6-trinitrotoluene by *Serratia marcescens*. *Biotechnology Letters*. 19 (3): 291-294.
- Pérez SR, Ábalos RA, Gómez M and Cantero M. 2006. Biodegradation of crude oil by *Pseudomonas aeruginosa* AT18 strain. *Tecnología Química*. 27(1): 70-77.
- Raisz LG, Wiita B, Artis A, Bowen A, Schwartz S, Trahiotis M, Shoukri K, y Smith J. 1996. Comparison of the effects of estrogen alone and estrogen plus androgen on biochemical markers of bone formation and resorption in postmenopausal women. *J. Clinical Endocrinology Metabolism*. 81(1): 37-43.
- Rivas A, Granados A, Jiménez M, Olea F and Olea N. 2004. Exposición humana a disruptores endocrinos. *Ecosistemas*. 8 (003): 1-8.
- Shula J, Zhipei L, Zhipeng L and Haiyan R. 2009. Isolation and characterization of a bacterial strain that efficiently degrades sex steroid hormones. *Front Environ Sci Engin. China*. 1 (3): 325-328.
- Sigma Chemical Company, 1994. Material Safety Data Sheet. St. Louis, MO, USA 2p.
- Swan SH, Brazil C, Drobnis EZ, Fan Liu, Kruse RL and Hatch M. 2003. Geographic differences in semen quality of fertile U.S. males. En: Argemi F, Cianni N, Y Porta A. 2005. Disrupción endocrina: perspectivas ambientales y salud pública. *Toxicología Ambiental*. 39 (3): 291-300.
- Toft G, Hagmar L, Giwercman A, Bonde JP. 2004. Epidemiological evidence on reproductive effects

- of persistent organochlorines in humans. *Reproductive Toxicology*. 19: 5-26.
- Yamakata H, Moriyoshi, Ohmoto T, Ohe T and Sakai K. 2007. Degradation of Bisphenol A by *Bacillus pumilus* Isolated from Kimchi, a Traditionally Fermented Food. *Applied Biochemistry and Biotechnology*. 1 (36): 39-51. *Environmental Science y Technology*.
- Yang YY, Pereyra LP, Young RB, Reardon KF and Borch T. Testosterone-Mineralizing Culture Enriched from Swine Manure: Characterization of Degradation Pathways and Microbial Community Composition. *45* (16): 6879–6886.
- Walaa AF, Abbas IH, Elwan KM, Swellun MA and Dougdoug KhA. 2009. Biotransformation of Progesterone by Microbial Steroids. *Journal of Applied Sciences Research*. 5 (1): 137-143.
- Weber S, Leuschner P, Kämpfer P, Wolfgang D and Hollender J. 2004. Degradation of estradiol and ethinyl estradiol by activated sludge and by a defined mixed culture. *Applied Microbiology and Biotechnology*. 67:106-112.