Supporting Information

A Novel Recirculating Aquaculture System for Sustainable Aquaculture: Enabling Wastewater Reuse and Conversion of Waste-to-Immune-Stimulating Fish Feed

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Supporting Information: 16 pages total including text describing physicochemical analysis, 2 tables, 5 figures, and economic analysis

Table of Content

Physicochemical Analysis. The COD of AW or growth media was determined according to standard methods (with potassium dichromate in sulfuric acid) using a CHEMetrics Inc. mercury-free COD Test Kit, 0–1,500 ppm (HR). To determine total nitrogen-nitrogen (TN-N) in AW and NH_4^+-N in media, the samples were first filtered (0.45-µm) and wet-digested using peroxo-disulphate, before being analyzed according to standard methods (HACH assay kits, TNT 826 and 828). The *Z. denitrificans* ZD1 growth and supernatant absorbance were monitored based on absorbance (OD_{600}) using a UV–visible scanning spectrophotometer (VWR, 3100 PC). The CDW was measured after centrifuging the collected sample at the stationary phase using a Sorvall Legend XTR centrifuge (4,500 × g for 10 min at 4°C). Subsequently, the pellets were washed with DI water and desiccated at 105°C in pre-weighed glass tubes. The cell concentration was determined by dividing the weight difference (before and after drying) by the sample volume. To determine the PHB content, the dried biomass was digested with 1 mL of concentrated H_2SO_4 at 70°C for 4 h and then neutralized by adding 4 M NaOH. The digested solution was centrifuged, and crotonic acid was determined at 235-nm against that of pure PHB standards treated alike.

Table S1. Characteristics of agro-industrial wastewaters used for the non-sterile production of PHB-rich *Z. denitrificans* ZD1 biomass

Organic Waste	COD (g/L)	TN (g-N/L)	Salinity (g/L)	pH
AW	0.205	0.025	3	8.2
Glycerol	12.2	-	30	7.4
CWW	50.4	1.47	0	6.5

Note: AW = Aquaculture wastewater (i.e., fish tank effluent)
CWW = Cheese production wastewater

Table S2. Comparison of Biomass Composition in this Study with other Protein Sources.

Protein Source		Energy Source/Substrate	PHB (%)	Protein[a] (%)	Lipid (%)	Ash (%)	Energy (MJ/kg)	References
Microbial biomass (SCPs)	*Z. denitrificans* ZD1	Glycerol	48	45.5	50.4	4.1	23.4	This study
		CWW	12	34.8	13.6	51.6	11.2	
	Bacillus licheniformis	Potato processing waste		38		11		[1]
	Purple phototrophic bacteria	Light/Poultry WW[b]		~75[c]	~20		22	[2]
		Light/Dairy WW		~61	~29			
		Light/Sugar WW		~42	~20			
	Methane-oxidizing bacteria	Biogas methane	43-73	60	8-11	6-9		[3-5]
	Hydrogen-oxidizing bacteria	Hydrogen	57	75	-			[3]
	Microalgae: *Chlorella vulgaris* and *Scenedesmus* species	Light/Poultry WW		~65	~27			[2]
		Light/Dairy WW		~37	~59			
		Light/Sugar WW		~14	~15			
	Yeast	Organic carbon		45-55	1-6	5-10	19.9	[6, 7]
Fishmeal		-		63	11	16	20.1	[8]
Soybean meal		-		44	2.2	5	21.3	[8]

Notes: Results are expressed as dry-weight basis and presented as mean values from triplicate experiments. [a] The protein content in this study was adjusted (1–5%) to reduce the overestimation caused by the interference with the non-protein nitrogen compounds using the Dumas method. [b] WW refers to wastewater. [c] The values were estimated from Figure 3 in Hülsen et al.[2]

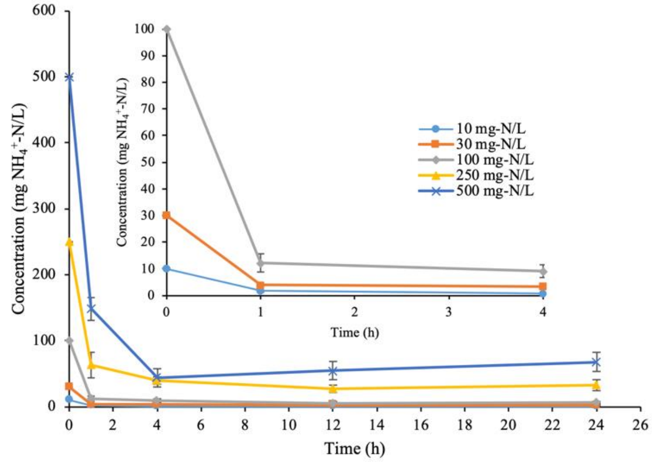

Figure S1. Change in NH_4^+-N concentration with time after adsorption by natural zeolite under different initial NH_4^+-N concentrations.

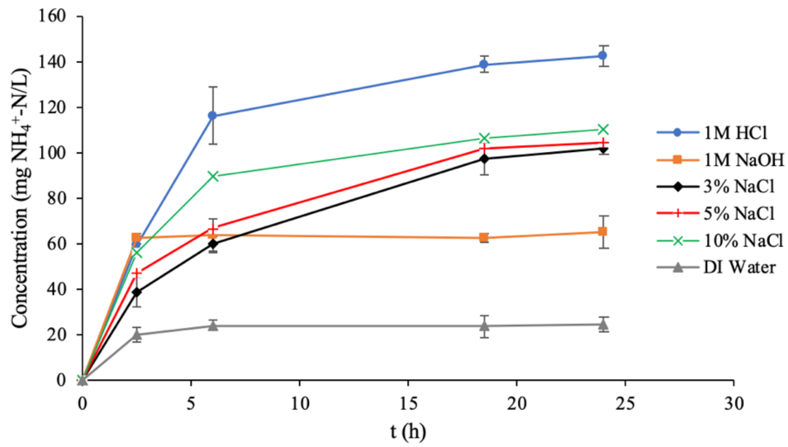

Figure S2. Concentration of released NH_4^+-N from ammonium-laden zeolite using various desorption solutions.

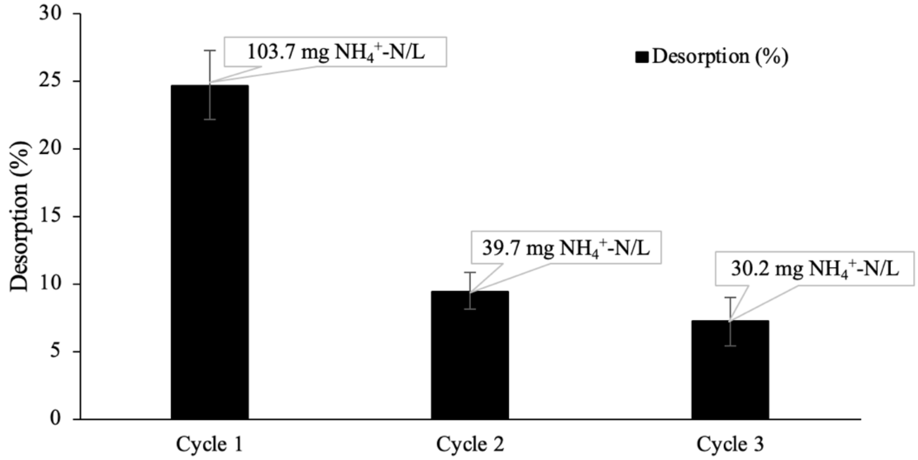

Figure S3. Desorption (%) from each cycle after extraction of ammonium-laden zeolite with 3% NaCl. NH_4^+-N concentration detected in the extract after each cycle was shown on the top of the bar.

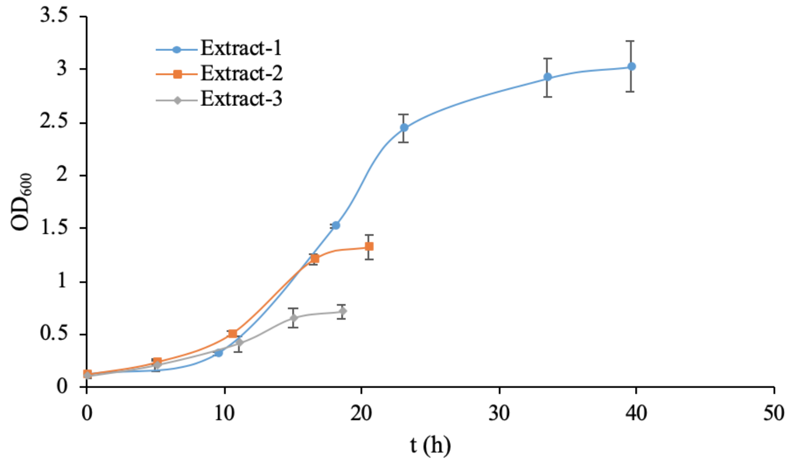

Figure S4. *Z. denitrificans* ZD1 cultivation in N-free MSM, glycerol (5 g/L), and one of the three extracts obtained from spent zeolite after three cycles of 3% NaCl desorption.

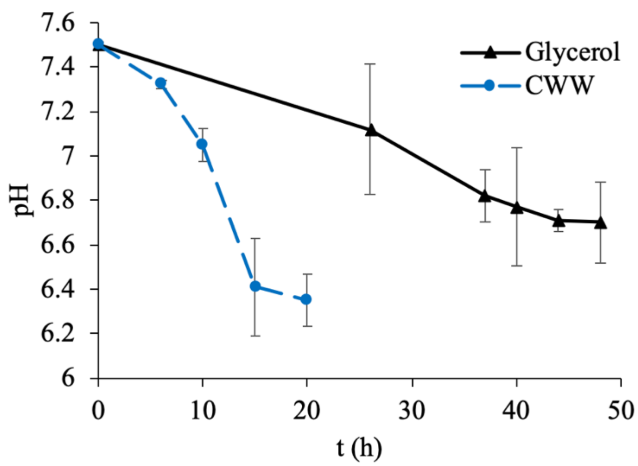

Figure S5. Changes in pH during the growth of *Z. denitrificans* ZD1 in agro-industrial wastes/wastewaters, glycerol and CWW.

Economic Analysis

The assumptions and calculations for computing the annual production cost of farmed fish using the conventional RAS or the proposed RAS-PHB is provided in this section. The economic analysis was separated into eight scenarios; four scenarios for each RAS based on the farmed fish species (tilapia (T) and red drum (R)). Scenarios TA and TB and Scenarios RA and RB represent the conventional RAS with the supplementation of antibiotics or pure commercial PHB, respectively, to achieve the same overall tilapia and red drum production. Scenarios TC and TD and Scenarios RC and RD represent RAS-PHB with the supplementation of glycerol- or CWW-grown PHB-rich *Z. denitrificans* ZD1 biomass, respectively, as alternatives.

The following assumptions were made for computing production cost for all scenarios:

Assumptions applied to all scenarios:

- An annual production of 500 ton of farmed tilapia or red drum using RAS.
- Volume of aquaculture system is 1000 m^3
- Stock density is 50 kg fish/m^3
- The aeration energy required for *Z. denitrificans* ZD1 growth in the bioreactor is similar to that required for the conventional biofilter in RAS.
- The cost of the zeolite filter is similar to the sand filter in RAS.
- Feed cost is \$0.18/lb = \$0.40/kg feed.[9]
- The average food conversion ratio for tilapia and red drum is 2.2[10, 11] and 2.0 kg feed/kg fish produced,[12] respectively.
- The protein content of a typical tilapia and red drum feed is 30%[13] and 40%,[14, 15] respectively.
- The application of antibiotics, commercial pure PHB, or PHB-rich *Z. denitrificans* ZD1 exhibits similar fish survival rates and the final kg of fish produced.

Assumptions applied to Scenarios TA and TB and Scenarios RA and RB:

- Average daily water replacement in the conventional RAS is 10% of the water volume.[9]
- Water replacement cost is ~\$0.32/m^3.[11]
- Average solid waste produced in tilapia culture is 230 kg/ton fish produced = 0.25 kg/kg fish produced.[16]
- Average solid waste produced in red drum culture is ~ 0.31 kg/kg fish produced.[17]
- Solid waste disposal cost is ~\$0.62/kg of waste. Such estimate is based on \$8726 annual operating cost for 127 mt of solid waste.[18]
- **For Scenario TA and RA only:**
    - Antibiotics use in the conventional RAS is 0.53 kg/ton.[19]
    - Average cost of antibiotics is \$150/kg.[20]
    - The survival rate of fish receiving 75 mg dose/kg bodyweight/day of oxytetracycline antibiotic is 85%.[21]
- **For Scenario TB and RB only:**
    - Pure commercial PHB use in the feed (5% w/w).[22-24]

- Commercial PHB price is \$0.48/kg.[25]
- The survival rate of fish receiving 5% (w/w) PHB in the feed is 85%.[24]

Assumptions applied to Scenarios TC and TD and Scenarios RC and RD:

- The PHB-rich *Z. denitrificans* ZD1 biomass to be used in the proposed RAS-PHB is 60% of the feed.
- Chitosan dosage of 50 mg/L is used to harvest 80% of biomass.
- Price of chitosan is \$7/kg.[26]
- **For Scenario TC and RC only:**
  - Glycerol is the organic waste to cultivate *Z. denitrificans* ZD1.
  - Price of glycerol is \$0.04/kg.[27]
- **For Scenario TD and RD only:**
  - Cheese whey wastewater (CWW) is the organic waste to cultivate *Z. denitrificans* ZD1.
  - Although CWW is considered a dairy waste that can be obtained at no cost, its price assumed to be €25/ton = \$0.028/kg.[28]

Production Cost of Tilapia Farming using Conventional RAS (Scenarios TA and TB)

(i) Annual feed cost for producing 1 kg of fish:

➔ 2.2 kg feed/kg fish produced × \$0.40/kg feed = \$0.9/kg fish produced

(ii) Annual water replacement cost for producing 1 kg fish:

➔ 0.1 m^3 water replaced/m^3 water volume/d × 1000 m^3 water volume × 365 d/yr × \$0.32/m^3 = \$11680/yr

➔ \$11680/yr /(500 ton/yr) = \$24/ton = \$0.024/kg fish produced

(iii) Annual solid waste disposal cost for producing 1 kg of fish:

➔ 0.25 kg solid waste/kg fish produced × \$0.62/kg solid waste = \$0.15/kg fish produced

(iv) Annual antibiotics/ pure commercial PHB cost for producing 1 kg fish:

➔ **Scenario TA – Use of antibiotics:** 0.53 kg antibiotics/ton fish produced × 1 ton fish/1000 kg fish × \$150/kg antibiotics = \$0.08/kg fish produced

➔ **Scenario TB – Use of pure commercial PHB:** 2.2 kg feed/kg fish produced × 0.05 kg PHB/kg feed × \$4.4/kg PHB = \$0.48/kg fish produced

Scenario TA: Total cost = \$0.9 + \$0.024 + \$0.15 + \$0.08 = \$1.2/kg fish produced/yr

Scenario TB: Total cost = \$0.9 + \$0.024 + \$0.15 + \$0.48 = \$1.6/kg fish produced/yr

Production Cost of Red Drum Farming using Conventional RAS (Scenarios RA and RB)

(v) Annual feed cost for producing 1 kg of fish:

➔ 2.0 kg feed/kg fish produced × \$0.40/kg feed = \$0.8/kg fish produced

(vi) Annual water replacement cost for producing 1 kg fish:

➔ 0.1 m^3 water replaced/m^3 water volume/d × 1000 m^3 water volume × 365 d/yr × \$0.32/m^3 = \$11680/yr

➔ \$11680/yr /(500 ton/yr) = \$24/ton = <u>\$0.024/kg fish produced</u>

(vii) Annual solid waste disposal cost for producing 1 kg of fish:

➔ 0.31 kg solid waste/kg fish produced × \$0.62/kg solid waste = <u>\$0.20/kg fish produced</u>

(viii) Annual antibiotics/ pure commercial PHB cost for producing 1 kg fish:

➔ **Scenario RA – Use of antibiotics:** 0.53 kg antibiotics/ton fish produced × 1 ton fish/1000 kg fish × \$150/kg antibiotics = <u>\$0.08/kg fish produced</u>

➔ **Scenario RB – Use of pure commercial PHB:** 2.2 kg feed/kg fish produced × 0.05 kg PHB/kg feed × \$4.4/kg PHB = <u>\$0.48/kg fish produced</u>

Scenario RA: Total cost = \$0.8 + \$0.024 + \$0.20 + \$0.08 = \$1.1/kg fish produced/yr
Scenario RB: Total cost = \$0.8 + \$0.024 + \$0.20 + \$0.48 = \$1.5/kg fish produced/yr

<u>Production Cost of Tilapia Farming using Proposed RAS-PHB (Scenarios TC and TD)</u>

According to De Schryver et al., in a conventional RAS, tilapia can be produced with an average food conversion ratio of 2.2 kg feed/kg fish and 30% protein content in the regular feed.[11] In the proposed RAS-PHB, 60% of the regular fish feed is assumed to be replaced by PHB-rich *Z. denitrificans* ZD1 biomass that was produced from glycerol (Scenario TC) or CWW (Scenario TD). The protein contents of glycerol- and CWW-grown *Z. denitrificans* ZD1 were 45.5% and 34.8%, respectively. Therefore, the amount of *Z. denitrificans* ZD1 biomass needs to be added to the feed to produce 1 kg of fish is:

➔ 0.3 kg protein/kg feed × 2.2 kg feed/kg fish produced = 0.66 kg protein/kg fish produced

➔ **Scenario TC – Use of glycerol:** (0.66 kg protein/kg fish produced × 0.60) /(0.455 kg protein/kg ZD1 biomass) = 0.87 kg ZD1 biomass/kg fish produced

➔ **Scenario TD – Use of CWW:** (0.66 kg protein/kg fish produced × 0.60) /(0.348 kg protein/kg ZD1 biomass) = 1.13 kg ZD1 biomass/kg fish produced

(i) Total feed cost for producing 1 kg fish in RAS-PHB = Cost of 40% regular feed + Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

➔ Cost of 40% regular feed: 0.40 × (2.2 kg feed/kg fish produced × \$0.40/kg feed) = <u>\$0.36/kg fish produced</u>

➔ Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

⇒ **Scenario TC – Use of glycerol:** Cost to produce 0.87 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (1.97 g ZD1 biomass/L)/(10 g glycerol/L) = 0.20 g ZD1 biomass/g glycerol = 5 kg glycerol/kg ZD1 biomass
- 0.87 kg ZD1 biomass/kg fish produced × 5 kg glycerol/kg ZD1 biomass = 4.35 kg glycerol/kg fish produced
- 4.35 kg glycerol/kg fish produced × \$0.04/kg glycerol = <u>\$0.17/kg fish produced</u>
- The total feed cost = \$0.36 + \$0.17 = <u>\$0.53/kg fish produced</u>

⇒ **Scenario TD – Use of CWW:** Cost to produce 1.0 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (3.24 g ZD1 biomass/L)/(8 g CWW/L) = 0.4 g ZD1 biomass/g CWW = 2.4 kg CWW/kg ZD1 biomass
- 1.13 kg ZD1 biomass/kg fish produced × 2.4 kg CWW/kg ZD1 biomass = 2.7 kg CWW/kg fish produced
- 2.7 kg CWW/kg fish produced × \$0.028/kg CWW = <u>\$0.08/kg fish produced</u>
- The total feed cost = \$0.36 + \$0.08 = <u>\$0.44/kg fish produced</u>

Note: In the conventional RAS, the amount of pure PHB supplied in the feed is 0.05 kg PHB/kg feed. In the proposed RAS-PHB, the amount of PHB supplemented through PHB-rich ZD1 biomass is:

➔ **Scenario TC – Use of glycerol:**

⇒ 0.87 kg ZD1 biomass/kg fish produced × 0.48 kg PHB/kg ZD1 biomass = 0.42 kg PHB/kg fish produced

⇒ 0.42 kg PHB/kg fish produced × 1 kg fish produced/2.2 kg feed = 0.19 kg PHB/kg feed

⇒ (0.19 kg PHB/kg feed)/(0.05 kg PHB/kg feed) = 3.8 times higher than the needed pure PHB in the conventional RAS, which is also the amount required to promote growth and survival of Nile tilapia.[24]

➔ **Scenario TD – Use of CWW:**

⇒ 1.13 kg ZD1 biomass/kg fish produced × 0.12 kg PHB/kg ZD1 biomass = 0.14 kg PHB/kg fish produced

⇒ 0.14 kg PHB/kg fish produced × 1 kg fish produced/2.2 kg feed = 0.06 kg PHB/kg feed

⇒ (0.06 kg PHB/kg feed)/(0.05 kg PHB/kg feed) = 1.2 times higher than the needed pure PHB in the conventional RAS.

(ii) The chitosan coagulant cost:

➔ **Scenario TC – Use of glycerol:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (1.97 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 31.5 kg ZD1 biomass/kg chitosan = 0.032 kg chitosan/kg ZD1 biomass

⇒ 0.032 kg chitosan/kg ZD1 biomass × 0.87 kg ZD1 biomass/kg fish produced = 0.028 kg chitosan/kg fish produced

⇒ 0.028 kg chitosan/kg fish produced × $7/kg chitosan = <u>$0.19/kg fish produced</u>

➔ **Scenario TD – Use of CWW:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (3.24 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 51.8 kg ZD1 biomass/kg chitosan = 0.02 kg chitosan/kg ZD1 biomass

⇒ 0.02 kg chitosan/kg ZD1 biomass × 1.13 kg ZD1 biomass/kg fish produced = 0.02 kg chitosan/kg fish produced

⇒ 0.02 kg chitosan/kg fish produced × $7/kg chitosan = <u>$0.14/kg fish produced</u>

Scenario TC: Total cost = $0.36 + $0.17 + $0.19 = $0.7/kg fish produced Scenario TD: Total cost = $0.36 + $0.08 + $0.14 = $0.6/kg fish produced

<u>*Production Cost of Red Drum Farming using Proposed RAS-PHB (Scenarios RC and RD)*</u>

Red drum can be produced with an average food conversion ratio of 2.0 kg feed/kg fish[12] and 40% protein content in the regular feed.[14, 15] Therefore, the amount of *Z. denitrificans* ZD1 biomass needs to be added to the feed to produce 1 kg of fish is:

➔ 0.4 kg protein/kg feed × 2.0 kg feed/kg fish produced = 0.8 kg protein/kg fish produced

➔ **Scenario RC – Use of glycerol:** (0.8 kg protein/kg fish produced × 0.60) /(0.455 kg protein/kg ZD1 biomass) = 1.05 kg ZD1 biomass/kg fish produced

➔ **Scenario RD – Use of CWW:** (0.8 kg protein/kg fish produced × 0.60) /(0.348 kg protein/kg ZD1 biomass) = 1.38 kg ZD1 biomass/kg fish produced

(i) Total feed cost for producing 1 kg fish in RAS-PHB = Cost of 40% regular feed + Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

➔ Cost of 40% regular feed: 0.40 × (2.0 kg feed/kg fish produced × $0.40/kg feed) = <u>$0.32 kg fish produced</u>

➔ Cost to produce kg ZD1 biomass/kg fish produced based on the organic waste type

⇒ **Scenario RC – Use of glycerol:** Cost to produce 1.05 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (1.97 g ZD1 biomass/L)/(10 g glycerol/L) = 0.20 g ZD1 biomass/g glycerol = 5 kg glycerol/kg ZD1 biomass
- 1.05 kg ZD1 biomass/kg fish produced × 5 kg glycerol/kg ZD1 biomass = 5.3 kg glycerol/kg fish produced
- 5.3 kg glycerol/kg fish produced × $0.04/kg glycerol = <u>$0.21/kg fish produced</u>
- The total feed cost = $0.32 + $0.21 = <u>$0.53/kg fish produced</u>

⇒ **Scenario RD – Use of CWW:** Cost to produce 1.38 kg ZD1 biomass/kg fish produced.

- ZD1 biomass yield = (3.24 g ZD1 biomass/L)/(8 g CWW/L) = 0.4 g ZD1 biomass/g CWW = 2.4 kg CWW/kg ZD1 biomass
- 1.38 kg ZD1 biomass/kg fish produced × 2.4 kg CWW/kg ZD1 biomass = 3.31 kg CWW/kg fish produced
- 3.31 kg CWW/kg fish produced × \$0.028/kg CWW = <u>\$0.093/kg fish produced</u>
- The total feed cost = \$0.32 + \$0.093 = <u>\$0.41/kg fish produced</u>

(ii) The chitosan coagulant cost:

➔ **Scenario RC – Use of glycerol:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (1.97 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 31.5 kg ZD1 biomass/kg chitosan = 0.032 kg chitosan/kg ZD1 biomass

⇒ 0.032 kg chitosan/kg ZD1 biomass × 1.05 kg ZD1 biomass/kg fish produced = 0.033 kg chitosan/kg fish produced

⇒ 0.033 kg chitosan/kg fish produced × \$7/kg chitosan = <u>\$0.24/kg fish produced</u>

➔ **Scenario RD – Use of CWW:**

⇒ Required chitosan amount = 0.80 kg ZD1 biomass harvested/kg ZD1 biomass × (3.24 g ZD1 biomass/L) × 1 L/0.05 g chitosan = 51.8 kg ZD1 biomass/kg chitosan = 0.02 kg chitosan/kg ZD1 biomass

⇒ 0.02 kg chitosan/kg ZD1 biomass × 1.38 kg ZD1 biomass/kg fish produced = 0.03 kg chitosan/kg fish produced

⇒ 0.03 kg chitosan/kg fish produced × \$7/kg chitosan = <u>\$0.21/kg fish produced</u>

Scenario RC: Total cost = \$0.32 + \$0.21 + \$0.24 = \$0.8/kg fish produced
Scenario RD: Total cost = \$0.32 + \$0.093 + \$0.21 = \$0.6/kg fish produced

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