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Copper Nanoparticle Inhibition of Fermentative Hydrogen Yield by *Enterobacter cloacae*: Nonlinear Model Discrimination Identifies a Sharp Wang-Type Threshold

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Editorial

Copper nanoparticles (CuNPs) may enhance anaerobic bioprocesses at trace concentrations but suppress microbial metabolism when exposure exceeds cellular tolerance. This study re-analysed published hydrogen-yield data for *Enterobacter cloacae* exposed to 0-12.5 mg/L phyto-genic CuNPs and compared seven nonlinear inhibition models using goodness-of-fit, information-theoretic, predictive-bias, and multi-criteria indices. Hydrogen yield remained near 26-29 mol H₂/mol glucose through 7.5 mg/L, then fell to approximately 12 and 5 mol H₂/mol glucose at 10 and 12.5 mg/L, respectively. The Wang model described this abrupt transition most effectively ($R^2=0.99$; adjusted $R^2=0.98$; RMSE=1.26; bias factor=0.97; accuracy factor=1.07) and ranked first by Multiobjective Optimization by Ratio Analysis (MOORA score=0.82). Its estimated maximum yield was 28.690 mol H₂/mol glucose (95% CI: 26.336-31.044), the characteristic inhibitory concentration KC was 9.686 mg/L (8.969-10.403), and the response-shape exponent m was 7.157 (3.699-10.614). The Shukor model ranked second (MOORA score=0.24; adjusted $R^2=0.89$; RMSE=2.75). These results reveal a steep concentration threshold rather than gradual proportional inhibition. For process control, CuNP exposure should remain well below the estimated transition region, while validation with independent datasets, dissolved-copper measurements, nanoparticle characterization, and uncertainty-aware dose-response experiments is required before scale-up under realistic reactor operating conditions.

INTRODUCTION

Hydrogen is an attractive energy carrier because its use can avoid carbon dioxide formation at the point of conversion, although the environmental merit of any hydrogen pathway depends on its feedstock, energy source, and system boundary. Biological hydrogen production is especially relevant where renewable energy recovery can be coupled to treatment of carbohydrate-rich wastes and industrial effluents. Among the available biological routes, dark fermentation operates without light, accepts diverse substrates, and proceeds at comparatively high volumetric rates [1-5]. Its practical limitation is that metabolic products such as acetate, butyrate, lactate, ethanol, and reduced cofactors divert substrate electrons away from hydrogen, so reactor performance is highly sensitive to community structure and operating

conditions [3,6-9]. Facultative anaerobes of the genus *Enterobacter* are useful hydrogen producers because they grow rapidly, consume oxygen that enters the reactor, and express formate hydrogen-lyase under anaerobic conditions. *Enterobacter cloacae* has produced hydrogen from glucose and several waste-derived hydrolysates, while temperature, pH, substrate concentration, nitrogen availability, and metabolite accumulation alter its yield and rate [10-14]. The organism's robustness does not remove the need for toxicity control.

In real feedstocks, trace metals and engineered particles may enter through industrial wastewater, catalysts, antimicrobial coatings, process additives, or corrosion. Their effects may be stimulatory at low dose and inhibitory at higher dose, creating a non-monotonic operational window that cannot be represented adequately by a single linear coefficient [15-18]. Nanomaterials

have therefore acquired a dual role in anaerobic biotechnology. Iron, nickel, magnetite, carbon-based materials, and conductive composites can improve electron transfer, provide enzyme cofactors, or support biomass retention, and several studies report enhanced hydrogen production after carefully controlled dosing [19-24]. Copper nanoparticles are more difficult to manage. Copper is an essential micronutrient, yet CuNPs and released Cu ions can damage membranes, disturb metal homeostasis, bind protein thiols, and promote reactive oxygen species. Toxicity depends on particle size, coating, aggregation, medium composition, redox state, dissolution, and the physiology of the exposed organism [15-18,25-28]. Consequently, nominal CuNP concentration is an operational exposure indicator rather than a complete mechanistic dose metric.

Mohanraj et al. examined phytogenic CuNPs during fermentative hydrogen production by *E. cloacae* and *Clostridium acetobutylicum* and reported marked suppression at the upper concentrations [29]. The concentration-response profile for *E. cloacae* is notable because the hydrogen yield changes little across the lower doses before declining sharply. Such a threshold-like pattern has direct engineering importance: a reactor may appear stable during incremental contamination but lose much of its hydrogen-forming capacity over a narrow additional dose interval. A model that identifies the location and steepness of this transition can support alarm limits, sensitivity analysis, and experimental design, provided its parameters are not mistaken for universally transferable toxicological constants.

The distinction between hydrogen yield and microbial growth rate is important in this setting. A fall in molar hydrogen yield may arise from reduced viability, altered glucose conversion, redistribution of fermentation products, direct impairment of hydrogen-forming enzymes, or a combination of these responses. Yield data alone cannot separate those pathways. Nevertheless, a well-fitted empirical inhibition curve remains valuable because it summarizes the net process response that determines gas recovery. Its most defensible role is to locate concentrations requiring closer monitoring and to guide the placement of additional experimental doses, not to substitute for measurements of biomass, substrate consumption, copper speciation, or metabolic products.

This modelling perspective is especially relevant during scale-up, when small changes in contaminant loading can propagate into substantial losses of recoverable gas. Nonlinear inhibition models encode different assumptions about how performance declines. Andrews-type and exponential expressions generally impose gradual curvature, whereas critical-concentration or flexible power models can represent a delayed, steep response. Wang et al. proposed a sigmoidal inhibition expression for hydrogen production under high acetate exposure, and subsequent reviews highlighted the value of fitting inhibition data with models suited to the observed response geometry [30,31]. Model choice should not rest on R2 alone. Residual magnitude, parameter penalty, systematic bias, multiplicative accuracy, and numerical stability each address a different aspect of fit [32-38]. Information criteria are particularly useful for balancing fit against model complexity, while multi-criteria decision tools such as MOORA can integrate metrics that otherwise nominate different winners [39,40].

The present study re-analysed the published *E. cloacae*-CuNP concentration-response dataset using seven inhibition equations: Wang, modified Han-Levenspiel, modified Andrews, Shukor, Amor, Liu, and Kai models. The objectives were to (i) determine which equation best represents the observed threshold

response; (ii) estimate interpretable yield and inhibition parameters with 95% confidence intervals; (iii) compare models using complementary statistical indices and MOORA ranking; and (iv) clarify the process implications and limitations of the Wang model. The analysis is a secondary kinetic modelling study; it does not claim new nanoparticle synthesis or fermentation experiments. Its contribution lies in extracting a quantitative transition point and response shape from an existing dataset and placing those estimates in a form useful for designing confirmatory experiments and CuNP exposure controls.

MATERIALS AND METHODS

Data source and response variable

The concentration-response values were obtained from the *E. cloacae* experiment reported by Mohanraj et al. [29]. The analysed series comprised nominal CuNP concentrations of 0, 2.5, 5.0, 7.5, 10.0, and 12.5 mg/L. The response was hydrogen yield (mol H₂/mol glucose), as presented in the source dataset. No new biological experiment was conducted. Graphical values were treated as the experimental observations used in the supplied modelling file; therefore, conclusions apply to this dataset and its reported exposure conditions.

Inhibition models

Seven nonlinear equations were evaluated. In the expressions below, $Y(C)$ is hydrogen yield at CuNP concentration C , Y_{max} is the uninhibited or fitted maximum yield, K_C or K_i is a characteristic inhibition concentration, C_{crit} (or S_m) is the fitted concentration associated with complete inhibition in critical-concentration models, and m , n , or k is a dimensionless shape parameter. The remaining equations were implemented as specified in the original modelling worksheet and their cited formulations [30,31,41-44]. Because different parameterizations can share a model name, future reuse should report the complete equation rather than the name alone.

Effect of metal on growth rate of fermentative hydrogen production

The models (Table 1) utilized in this study is as follows;

Table 1. Inhibition models.

Models	Equation
Modified Han-Levenspiel	$r = u_{max} \left(1 - \frac{C}{C_{crit}}\right)^m$
Wang	$r = \frac{u_{max}}{1 + \left(\frac{C}{K_C}\right)^m}$
Liu	$r = \frac{u_{max} K_C}{K_C + C}$
Modified Andrews	$r = \frac{u_{max} C}{K_S + C + \left(\frac{C^2}{K_i}\right)}$
Shukor	$r = u_{max} \left(1 - \left(\frac{C}{S_m}\right)^n\right)$
Amor	$r = \frac{u_{max} C}{C + \left(\frac{C^2}{K_i}\right)}$
Kai	$r = u_{max} \exp(-kC)$

Where,

r is specific growth rate

μ_{max} is maximum specific growth rate

C is metal concentration

K_C and K_i are both concentration of heavy metal that is equal to half the maximum specific growth rate

K_i is the concentration of heavy metal that is equal to half the maximum specific growth rate
 C_{crit} and S_m is critical heavy metal concentration that causes a complete inhibition of specific growth rate
 n and k are the fitting constant

Fitting of the data

The Marquardt approach was employed to fit the nonlinear equations, and the CurveExpert Professional program (Version 1.6) was utilized. The algorithm aims to find the method that results in the minimum sum of squares between the predicted and measured values. The software automatically calculates the initial values using a method that considers the steepest ascent.

Statistical analysis

Parameters were estimated by nonlinear least squares with the Marquardt algorithm [32]. Fit was assessed using the coefficient of determination (R^2), adjusted R^2 , root-mean-square error (RMSE), Marquardt's percent standard deviation (MPSD), bias factor (BF), accuracy factor (AF), corrected Akaike information criterion (AICc), Bayesian information criterion (BIC), and Hannan-Quinn criterion (HQC) [33-38]. Higher adjusted R^2 was desirable; RMSE, MPSD, AICc, BIC, and HQC were minimized; and BF and AF were judged by proximity to 1. Confidence intervals were those produced by the nonlinear fit. With only six concentration levels, these intervals are model-based and should not be interpreted as external validation.

RMSE was calculated using the following formula;

$$RMSE = \sqrt{\frac{\sum_{i=1}^n (Pd_i - Ob_i)^2}{n-p}} \quad (\text{Eqn. 1})$$

BF and AF were calculated using the following formula;

$$\text{Bias factor} = 10 \left(\sum_{i=1}^n \log \frac{(Pd_i / Ob_i)}{n} \right) \quad (\text{Eqn. 2})$$

$$\text{Accuracy factor} = 10 \left(\sum_{i=1}^n \log \frac{|(Pd_i / Ob_i)|}{n} \right) \quad (\text{Eqn. 3})$$

AICc was calculated using the following formula;

$$AICc = 2p + n \ln \left(\frac{RSS}{n} \right) + \frac{2(p+1)+2(p+2)}{n-p-2} \quad (\text{Eqn. 4})$$

BIC was calculated using the following formula;

$$BIC = n \ln \left(\frac{RSS}{n} \right) + k \ln(n) \quad (\text{Eqn. 5})$$

HQC was calculated using the following formula;

$$HQC = n \ln \left(\frac{RSS}{n} \right) + 2k \ln(\ln n) \quad (\text{Eqn. 6})$$

Adjusted coefficient of determination (R^2) was calculated using the following formula;

$$\text{Adjusted } (R^2) = 1 - \frac{RMS}{S_y^2} \quad (\text{Eqn. 7})$$

$$\text{Adjusted } (R^2) = 1 - \frac{(1-R^2)(n-1)}{(n-p-1)} \quad (\text{Eqn. 8})$$

MPSD was calculated using the following formula;

$$MPSD = 100 \sqrt{\frac{1}{n-p} \sum_{i=1}^n \left(\frac{Ob_i - Pd_i}{Ob_i} \right)^2} \quad (\text{Eqn. 9})$$

MOORA ranking

The decision matrix was vector-normalized for each diagnostic. Adjusted R^2 was treated as beneficial, whereas error and information criteria were non-beneficial. For BF and AF, distance from unity was used conceptually as the desired direction. The normalized beneficial score was added and non-beneficial scores were subtracted to produce a MOORA score; a higher score indicated a stronger overall model [39,40]. Equal weighting was retained because no defensible, pre-specified hierarchy among the diagnostics was available.

RESULTS AND DISCUSSION

Concentration-response pattern

The experimental profile showed two distinct regions. From 0 to 7.5 mg/L CuNPs, hydrogen yield remained close to the upper plateau, at approximately 26-29 mol H_2 /mol glucose. At 10 mg/L, it decreased to about 12 mol H_2 /mol glucose, and at 12.5 mg/L it was approximately 5 mol H_2 /mol glucose (**Fig. 1**). Thus, the upper two concentrations corresponded to losses of roughly 59% and 83%, respectively, relative to the observed value near 29 mol H_2 /mol glucose at zero CuNP. The principal new result of the re-analysis is not merely that CuNPs inhibited hydrogen production, which was established in the source study [29], but that the inhibition geometry is sharply sigmoidal and can be localized quantitatively.

Model discrimination

The Wang equation was decisively superior on the principal residual measures (**Table 2**). It achieved $R^2=0.99$, adjusted $R^2=0.98$, and $RMSE=1.26$, compared with adjusted $R^2=0.89$ and $RMSE=2.75$ for the second-best Shukor model. Its BF of 0.97 indicates only slight overall underprediction, while $AF=1.07$ corresponds to a small average multiplicative deviation. The Wang model also gave the lowest BIC (3.94) and HQC (-1.01) among the compared equations. Visual inspection supports these statistics: its curve preserves the high-yield plateau through the lower CuNP range and then follows the rapid fall at 10-12.5 mg/L. Models imposing gradual decline began decreasing too early and consequently missed both the plateau and the terminal observations (**Figs. 1-7**).

AICc requires special caution here. Several reported AICc values favour simpler but visibly poorer two-parameter models, including Amor, because the sample size is only six and the small-sample penalty becomes severe when three parameters are fitted. The disagreement is informative rather than an error: no single criterion can simultaneously reward response-shape fidelity, low residual variance, and parsimony under such sparse sampling. MOORA combined the diagnostics and ranked Wang first (score 0.82), followed by Shukor (0.24); all other models had negative scores (**Table 3**). The ranking is therefore best viewed as structured evidence supporting Wang, not as proof that it will predict unseen experiments.

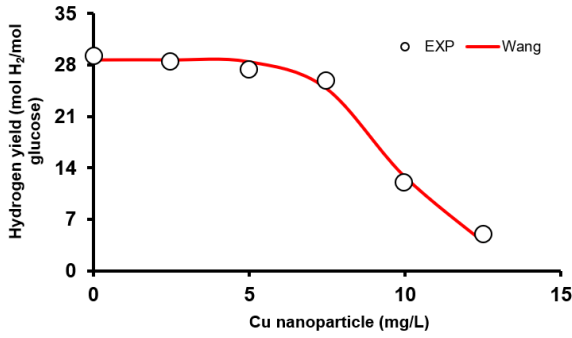


Fig. 1. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Wang model.

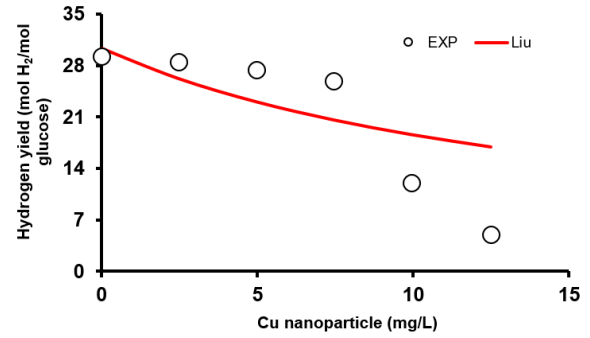


Fig. 5. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Liu model.

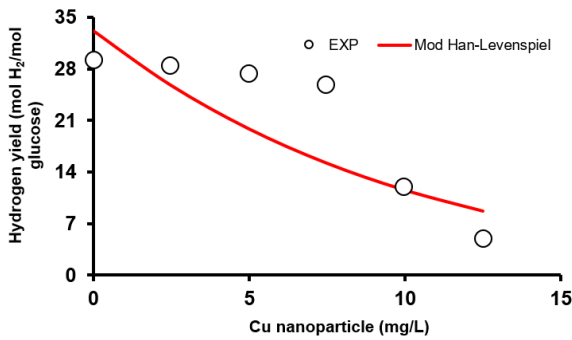


Fig. 2. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Modified Han-Levenspiel model.

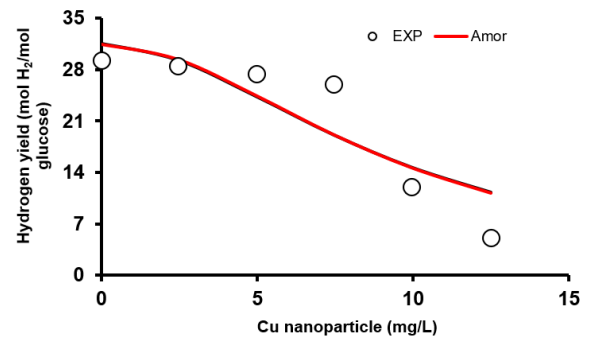


Fig. 6. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Amor model.

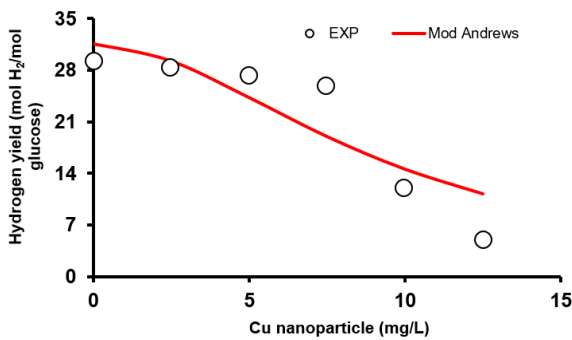


Fig. 3. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Modified Andrews model.

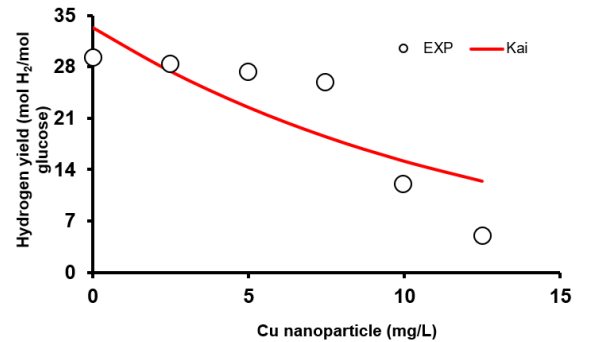


Fig. 7. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Kai model.

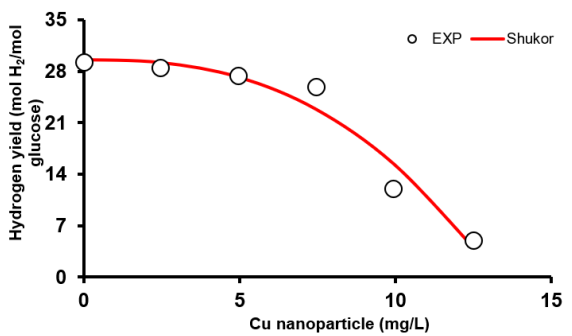


Fig. 4. The effect of copper nanoparticles on fermentative hydrogen production by *Enterobacter cloacae* as modelled using the Shukor model.

Table 2. Error function analysis for all models.

Model	<i>p</i>	MPSD	RMSE	R^2	adj R^2	AICc	BIC	HQC	BF	AF
Wang	3	125.51	1.26	0.99	0.98	52.57	3.94	-1.01	0.97	1.07
Mod Han-Levenspiel	3	829.40	8.29	0.52	0.21	75.23	26.60	39.26	0.95	1.32
Kai	2	634.36	6.34	0.47	0.12	41.74	23.32	38.70	1.12	1.36
Mod Andrews	3	598.76	5.99	0.67	0.18	71.32	22.69	23.36	0.20	7.07
Shukor	3	275.15	2.75	0.95	0.89	61.99	13.36	8.09	0.99	1.11
Amor	2	512.49	5.12	0.68	0.47	39.18	20.76	22.59	1.12	1.29
Liu	2	769.25	7.69	0.87	0.79	44.05	25.63	23.40	1.22	1.43

Note:
p no of parameter
adj R^2 adjusted correlation coefficient
RMSE Root mean square error
AF Accuracy factor
BF Bias factor
AICc corrected Akaike Information Criteria
MPSD Marquardt's percent standard deviation
n.a. not available

Table 3. MOORA ranking of models.

Model	MOORA Score	Rank
Wang	0.82	1
Shukor	0.24	2
Amor	-0.69	3
Liu	-1.02	4
Kai	-1.53	5
Mod Han-Levenspiel	-2.05	6
Mod Andrews	-2.29	7

Interpretation of the Wang model

The fitted Wang equation was $Y(C)=28.690/[1+(C/9.686)^{7.157}]$. Its Y_{max} estimate of 28.690 mol H₂/mol glucose (95% CI: 26.336-31.044) closely matches the observed low-dose plateau. K_C was 9.686 mg/L (8.969-10.403). Under this parameterization, K_C is the concentration at which the fitted response equals one-half of Y_{max} ; it is therefore an effective midpoint of inhibition, not a no-effect concentration and not necessarily an EC₅₀ measured by a formal toxicological protocol. At $C=K_C$, the model predicts approximately 14.35 mol H₂/mol glucose (Table 4).

Table 4. Parameter values for growth rate inhibition models.

Model	Value	95% Confidence Interval
Shukor		
μ_{max}	29.583	23.293 to 35.873
S_m	13.223	11.157 to 15.298
n	2.574	0.113 to 5.035
Wang		
μ_{max}	28.690	26.336 to 31.044
K_C	9.686	8.969 to 10.403
m	7.157	3.699 to 10.614

The exponent m controls the abruptness of the transition. The estimate $m=7.157$ is large, producing a nearly flat response at concentrations well below K_C and a steep descent around the midpoint. This explains why exponential and hyperbolic alternatives performed poorly: they distribute inhibition progressively across the entire concentration range, while the observations retain near-maximal yield until CuNP exposure approaches 8-10 mg/L. The 95% CI for m (3.699-10.614) is broad, reflecting limited concentration resolution around the inflection region. Even its lower bound, however, implies appreciable sigmoidicity. Additional measurements between 7.5 and 10.5 mg/L would reduce the covariance between K_C and m and test whether the apparent threshold is reproducible.

The Wang parameters also offer an operational interpretation. A nominal concentration near 9.7 mg/L marks the centre of the high-risk transition, not a safe operating ceiling. Process control should include a margin below this value because nanoparticle dissolution, aggregation, medium ligands, biomass concentration, and residence time can shift effective exposure [15-18,25-28]. Using the fitted equation only as a local interpolation, 80% of Y_{max} is retained at approximately 8.0 mg/L and 20% at approximately 11.8 mg/L. These derived values help define a confirmatory dose grid, but they should not be exported to other CuNP preparations or reactors without validation.

Comparison with the Shukor and other models

The Shukor equation reproduced the overall curvature and ranked second. It estimated $Y_{max}=29.583$ mol H₂/mol glucose (23.293-35.873), $S_m=13.223$ mg/L (11.157-15.298), and $n=2.574$ (0.113-5.035). Its fitted critical concentration is attractive for engineering interpretation, but the very wide confidence interval for n and the higher RMSE indicate weaker parameter precision and poorer local tracking than Wang.

The modified Han-Levenspiel, modified Andrews, Kai, Liu, and Amor equations did not reproduce the delayed collapse satisfactorily. Their adjusted R^2 values ranged from 0.12 to 0.79 and RMSE values from 5.12 to 8.29. BF and AF also revealed appreciable systematic or multiplicative error, most strikingly for modified Andrews ($B_F=0.20$; $A_F=7.07$). This comparison illustrates a general point in inhibition modelling: a plausible biological label does not ensure an appropriate mathematical response shape. Models developed for substrate inhibition, dissolved-metal stress, or metabolite accumulation may require re-parameterization when applied to nanoparticles, whose bioavailable fraction can change nonlinearly with nominal concentration.

Possible biological basis and process implications

The model is phenomenological and cannot identify a molecular mechanism. Nevertheless, a threshold response is compatible with several interacting processes. At lower exposure, extracellular ligands, cell envelopes, efflux systems, and intracellular copper-binding proteins may buffer Cu availability. Once those protective capacities are exceeded, increased dissolution or particle-cell contact can impair membrane integrity, disrupt iron-sulfur enzymes, bind thiol-containing proteins, and intensify oxidative stress [15-18,25-28]. Hydrogen formation in enteric bacteria depends on formate hydrogen-lyase and redox-balanced central metabolism [12,13]; disruption at multiple sites could therefore produce a steep loss of yield rather than a gradual decrease.

For biohydrogen systems, the result also tempers the common assumption that nanomaterial supplementation is inherently beneficial. Magnetite, nickel-based catalysts, and conductive carbon materials have enhanced electron transfer or hydrogen recovery in selected systems [19-24], yet performance is material-, dose-, organism-, and medium-specific. CuNP-containing streams should be monitored before blending with fermentable feedstocks, and mitigation should prioritize source control, separation, complexation assessment, or adsorption rather than relying on a fitted threshold alone. Reactor studies should measure dissolved Cu, particle size distribution before and after fermentation, oxidation state, extracellular polymeric substances, viable biomass, glucose conversion, metabolites, and hydrogenase-related activity. These measurements would distinguish true metabolic inhibition from particle settling, gas-measurement interference, or altered substrate utilization.

Limitations and validation priorities

This analysis contains six nominal concentrations from one published experiment, no independent test set, and no replicate-level residual structure. The impressive in-sample statistics may therefore overstate predictive performance. The original CuNP preparation and fermentation conditions constrain applicability, and nominal mg/L does not quantify the dissolved or cell-associated copper fraction. Future work should use randomized biological replicates, a no-particle ionic-copper control, a particle-only blank, at least four additional concentrations bracketing 8-11 mg/L, and external validation across batches. Bootstrap or profile-likelihood intervals, leave-one-concentration-out testing, and model averaging would provide more robust uncertainty estimates [33-38]. Mechanistic models could then link copper speciation, biomass, substrate conversion, and hydrogen formation rather than fitting yield alone.

CONCLUSION

The CuNP response of *E. cloacae* was characterized by a stable upper hydrogen-yield region followed by an abrupt decline between 7.5 and 12.5 mg/L. Among seven equations, the Wang model most faithfully represented this structure and ranked first by MOORA, with adjusted $R^2=0.98$, RMSE=1.26, KC=9.686 mg/L, and $m=7.157$. The fitted midpoint is useful for locating the transition but must not be interpreted as a universal safety threshold. The model should guide denser experiments around 8-11 mg/L and conservative CuNP control, followed by validation using independent batches and measurements of copper dissolution and speciation. This secondary analysis strengthens the quantitative interpretation of the published experiment while identifying the evidence required before reactor-scale application.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

AUTHORS' CONTRIBUTIONS

The conceptualization of the study was led by M.Y.S. Data analysis, review and editing was conducted by M.Y.S. and M.H.Y.

ETHICS APPROVAL

Not applicable. The study analysed food and feed commodities and involved no human participants or experimental animals.

DATA AVAILABILITY STATEMENT

Data availability: This study is a secondary nonlinear analysis of data reported by Mohanraj et al. [29]. No new experimental dataset was generated.

AI USAGE DECLARATION

During the preparation of this work, the author used OpenAI ChatGPT solely for language editing, grammatical correction, formatting support and refinement of readability. The author reviewed and edited the resulting text and take full responsibility for the accuracy, integrity, originality and final content of the publication.

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