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Modelling the kinetics of biomass and lactic acid production during Rohu fish pickle fermentation

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Abstract

Fish pickle was prepared from Rohu (*Labeo rohita*) by fermentation for 15 days and the changes in biomass growth, lactic acid production, and pH were evaluated. The data obtained were fitted in two most widely accepted microbial growth models: Modified Gompertz, and Logistic model and three well known lactic acid production models: Luedeking-Piret, Monteagudo et al., and Balannec et al. model for lactic acid fermentation. Model constants and coefficients were determined by a nonlinear regression method. All the models were validated using statistical parameters namely, coefficient of determination (R^2), root mean square error (RMSE), reduced chi-square (χ^2) and the reduced sum of squares (RSS). The results revealed that the viable cell counts increased from 0.91×10^7 cfu/ml to 9×10^9 cfu/ml after nine days of fermentation. The lactic acid increased by about 11.6 times in 12 days and remained constant for the rest of the fermentation period. The pH decreased from 6.5 to 4.2 on the 15th day of fermentation and then increased slightly till the final day of fermentation. The Logistic model and Luedeking-Piret model were best fitted to describe the biomass growth and lactic acid production by LAB during the fermentation period of pickle. The growth-associated and non-growth associated coefficients were determined to be 0.813 and 0.005, respectively. Based on these estimated parameters, it is concluded that lactic acid production in the fish pickle was a mixed type.

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1.0 Introduction

Fish pickle is a shelf-stable, intermediate moisture meat product, made up of spice mixture and oil/organic acid. Lactic acid build-up leads to a reduction of pH during the fermentation process thus providing better product quality, consistency, and prolonged stability. Pickle is a popular condiment in Nepal and India. Traditionally, it was used to ensure a stable food supply during times when fresh food would be unavailable, such as during the winter season or long voyage. Meat pickles are becoming a welcome addition to meals since they are ready-to-eat products with good shelf-stability at ambient temperature. Pickles are good appetizers and add variety to the daily diet. Pickles facilitate digestion by stimulating the flow of gastric juice. Different kinds of pickles contain varying amounts of nutrients depending upon the raw material and the method of preparation (Shrestha, 2001).

Fish meat is a good source of niacin, which is closely related to several metabolic reactions. Fish meat is also a moderately good source of riboflavin, thiamine that is needed for various body activities. They are rich in protein, vitamins, minerals, and polyunsaturated fatty acids. Fish pickles is considered highly nutritious because it contained high amount of protein, hence, it can help in solving the malnutrition problem (Shah, 2001). Fish pickles are also very popular due to its pleasant taste with a variety of production methods, from traditional to industrial processes are employed in India. The processing technique is simple and can be easily adopted by rural people or fisher-folk after short training. No expensive equipment is required and the overall investment is low (Devkate et al., 2015).

Lactic acid bacteria (LAB) are heterogeneous group of bacteria that plays a significant role in a variety of

fermentation processes. The bacteria ferment food carbohydrates and produce lactic acid as the main product of fermentation. Degradation of proteins and lipids and the production of various alcohols, aldehydes, acids, esters, and sulphur compounds contribute to the specific flavour development in different fermented food products. Lactic acid produced by LAB is a useful compound for food preservation because it maintains the acidic conditions of the fermented products, and is lethal to bacteria that cause food spoilage and food poisoning (Bintsis, 2018).

The fermentation of fish is most common in South-East Asia where fish is a major component in human diets. The fish variety, fermentation conditions, cure duration, and fermentation techniques are all affecting the texture, amino acid content, and volatile flavour profile of the finished product. To get a product with a pleasingly fragrant aroma and taste, very fresh fish must be used (Alcala et al., 2016). The purpose of fermentation modelling is to design large-scale fermentation processes using data obtained from small-scale fermentations. The mathematical models that are used to simulate a bioprocess can generally be classified as unstructured or structured. In unstructured models, the biomass is considered as one entity which is described only by its concentration. These models do not consider any changes that could take place in the inner cells. In structured models, the biomass is defined and includes intracellular components, such as the RNA content, enzymes, reactants and products. Unstructured models are mainly used to describe bacterial kinetics in complex natural substrates (Charalampopoulos et al., 2009). These models aim to mathematically relate the biochemical properties (response variables) to environmental factors (controlling factors), such as temperature, pH, water activity, and substrate composition. This contributes to a better understanding and control of the fermentation process. In general, modelling is performed in two stages; in the first stage the primary models that are applied to the experimental data describing the change of a response variable over time. In the second stage, secondary models are developed by expressing the bio-kinetic parameters derived from the primary models as a function of a single environmental factor. It must be noted that both primary and secondary models are built using data from experiments in synthetic media under carefully controlled conditions. The predictability of the models is then assessed in the complex food systems.

LAB research has focused so far on modelling the dependence of the growth rate on temperature and pH at pH-controlled conditions. Very little research has been done in the secondary modelling of growth when pH is not controlled or taking into account other bio-kinetic parameters, such as lactic acid and bacteriocins production (Vaazquez et al., 2008). The growth kinetic model has been based on the modified Gompertz model and logistic model for microbial growth and Leudeking and Piret model and Monteagudo et.al model for product formation. Modified forms of the Gompertz and logistic equations have been applied to indicate the effects of biomass, product and substrate inhibition on biomass growth for lactic acid production. Models including terms for both substrate and product inhibition have been suggested as well as models considering only product inhibition (Altiok et al., 2006). The kinetics of biomass production, pH and, lactic acid production during fermentation of fish pickle were analysed and the obtained results might assist in the process control and the prediction of shelf life ensuring the economic viability. The study will help to uncover critical factors of fermentation kinetics of fish pickle that many researchers were unable to explore.

2.0 Methodology

2.1 Material

Fresh fish was purchased from Regional Agricultural Research Station, Tarahara, Sunsari, Nepal (26.7056° N, 87.2569° E). It was brought by keeping in a clean polythene bag within 20 minutes in the laboratory of the Central Campus of Technology, Dharan, Nepal. Spices including chili powder, turmeric powder, cumin, garlic, ginger, cloves, mustard oil, vinegar, black pepper, sugar, salt, tomato sauce were purchased from the local market of Dharan (26.8065° N, 87.2846° E) in Nepal.

2.2 Microbial analysis

Ten grams of the sample was taken and mixed with 90 ml of 0.85% w/v sterile physiological saline. After that blending was done for 5 minutes and the serial dilution was carried up to 10^{-8}/ml. Then it was plated by pour plate method in Lactobacillus MRS Agar–M641 supplemented with 1% $CaCO_3$ in triplicates. Finally, it was incubated at 30 °C in anaerobic gas pack system for 48–72 hours and the clear zone making colonies indicated the acid-producing bacteria which

were counted on the colony counter and expressed in terms of colony forming unit per gram (cfu/g) (Dewan and Tamang, 2007). Similarly, colonies of moulds and yeast were examined on PDA and YM agar supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulphate, respectively, and incubated aerobically at 28 °C for 72 h. Isolated colonies based on colony morphology were chosen randomly among the highest diluted plates. They were streaked again on the isolation media on fresh agar plates. The microscopic examination was conducted to verify the purity of the isolates (Tamang & Tamang, 2010).

2.3 Microbial characterisation and identification

A phase-contrast microscope was used to check the cell morphology of total bacterial isolates and their motility. LAB isolates were Gram-stained and tested for catalase production by placing a drop of 10% hydrogen peroxide solution on isolates, and were preliminarily identified based on carbon dioxide production from glucose, ammonia production from arginine, growth at different temperatures (15 °C, 37 °C, and 45 °C), the ability to grow in different concentrations of sodium chloride (1%, 3%, 5%, 7%, and 10%) and pH (3.9, 9.6) in MRS broth (M369, HiMedia, India) as described by Tamang & Pradhan (2019).

2.4 Analytical methods

The pH was determined directly using a digital pH meter and titratable acidity was expressed as a percentage of lactic acid of the sample (AOAC, 1990). The percentage of lactic acid was converted to a gram per litre as described by Khadka et al. (2010):

$$Concentration\ (\%) = \frac{Mass\ of\ solute\ (g)}{Volume\ of\ solvent\ (ml)} \times 100 \qquad (1)$$

2.5 Preparation of fish pickle

The fish pickle was prepared as described by Shikha et al. (2018). The procedure is outlined as follows:

i. Cut fish in pieces and repeatedly wash with tap water.

ii. Marinated with salt (3%), chili powder (4%) and turmeric powder (0.4%) and kept at room temperature (around 32 °C) for 30 min.

iii. Fried in mustard oil till brown, remove from heating and kept in room temp (around 32 °C).

iv. Onion (4%), garlic (16%), and ginger paste (2%) were fried till brown.

v. Cumin powder (2%), cloves (0.4%), salt (3%), sugar (10%), and vinegar (1%) were added to mixed spice paste under frying.

vi. Fried fish pieces were added to spice mixture and heated till the vinegar absorbed

vii. Tomato sauce (6%), tamarind (4%) and sodium benzoate as preservative were added

viii. Ready fish pickle was let cooled at room temp and packed in glass bottles.

2.6 Model parameters estimation

The non-linear least-squares regression was used to determine the kinetic parameters from nonlinear equations in microbial growth and product formation, and the least square method of curve fitting was used to fit the developed models (Mavituna & Sinclair, 2008). The error between observed and predicted values was minimised by adjusting the number of iterations in the system.

The solver add-in in Microsoft Excel 2013 was used to estimate and evaluate the parameters by fitting the experimental values to the proposed. The coefficient of determination (R^2), chi-square (χ^2), root mean square error (RMSE), mean absolute percentage error (MAPE) and residual sum of squares (RSS) of each mathematical model were calculated and a suitable model was chosen based on the goodness of fit with the highest value of R^2 and lowest value of χ^2, RMSE, MAPE, and RSS (Afolabi et al., 2015; Kaur et al., 2017).

2.7 Growth and fermentation kinetics modelling

The experimental biomass and lactic acid production were then compared with various, widely acceptable modelling equations. The applied microbial growth kinetics model is given in Table 1 and the product formation kinetics model is shown in Table 2.

To find the best suitable model to explain the fermentation behaviour of fish pickle, statistical tools were used as shown in Table 3

Table 1: Microbial growth kinetics model.

Model	Equations		Reference
Modified Gompertz	$\log\left(\frac{N}{N_0}\right) = A\exp\left\{-\exp\left[\frac{\mu_m}{A}(\lambda - t)+1\right]\right\}$	(2)	Kaushal et al. (2016)
Logistic	$X = \log_{10}\left(\frac{N}{N_0}\right) = \frac{A}{\left\{1+\exp\left[2+\left(\frac{4\mu_m}{A}\right)(\lambda - t)\right]\right\}}$	(3)	Guo et al. (2019)

Table 2: Product formation kinetics model.

Model	Equations		Reference
Leudeking and Piret	$\frac{dP}{dt} = m\frac{dX}{dt} + nX\left(1 - \frac{P}{P_{max}}\right)$	(4)	Rajasekar et al. (2015)
Monteagudo et al.	$\frac{dP}{dt} = m\frac{dX}{dt} + nX\left(1 - \frac{P}{P_{\max}}\right)$	(5)	Monteagudo et al.(1994)
Balannec et al.	$\frac{dP}{dt} = m\frac{dX}{dt} + nX\left(1 - \frac{[HL]}{[HI]_{inh}}\right)$	(6)	Bouguettoucha et al. (2008)

Table 3: Statistical tools used to test the goodness of fit.

Statistical tools	Equation		Reference
R^2	$\mathrm{RSS} = \sum_{i=1}^{N}\left(P_{exp,i} - P_{pre,i}\right)^2$	(7)	Neter et al. (1990)
χ^2	$\chi^2 = \sum_{i=1}^{N}\frac{\left(P_{exp,i} - P_{pre,i}\right)}{N-n}$	(8)	Ikonić et al. (2012)
RMSE	$\mathrm{RMSE} = \frac{1}{N}\sum_{i=1}^{N}\left(P_{exp,i} - P_{pre,i}\right)^2$	(9)	Olyaie et al. (2015)
RSS	$\mathrm{RSS} = \sum_{i=1}^{N}\left(P_{exp,i} - P_{pre,i}\right)^2$	(10)	Lahtinen et al. (2011)

Table 4: Statistical results of growth models.

Name	Parameters	R^2	χ^2	RMSE	RSS
Modified Gompertz	μ_m=0.47 h^{-1}, λ= 92.1 h and A=4.53	0.978	0.64	0.46	0.68
Logistic	μ_m= 0.21 h^{-1}, λ = 71.7 h and A=3.88	0.988	0.55	0.42	0.64

3.0 Results and discussion

Fish pickle was prepared and the changes in percentage acidity, pH, and microbial load were studied. Later, the modelling of rate equations for biomass (X) and lactic acid (P) was carried out to explain the fermentation process of a pickle. A set of non-linear algebraic equations comprising of the primary models was used to describe cell growth in terms of biomass (N) and lactic acid production (P) with time. The model parameters were estimated from the batch data of N and P vs. time. The parameters of the primary models were then fitted to the controlling factors (secondary models) by using linear or non-linear regression analysis.

3.1 LAB growth

The initial population of LAB in pickle was not significant. The initial logarithmic microbial load of 6.959 cfu/g peaked to 10 cfu/g at 12th day of the fermentation. The LAB population then decreased to 9.041 cfu/g at 15th day. The changes in the microbial load during natural fermentation of pickle is shown in Fig. 2.

The rapid growth of heterofermentative rods and homofermentative tetrads could be the cause of the initial exponential increase of the LAB population. Homofermentative strains of lactobacilli produce about 85% lactic acid from glucose and heterofermentative strains produce lactic acid, carbon dioxide, ethanol, and/or acetic acid in equimolar amounts (Lahtinen et al., 2011). The LAB population was nearly constant during the fermentation period of 6-9 days, it might be due to the disappearance of heterofermentative lactic and vigorous growth of *L. plantarum* and other homolactic during this period. The decrease in the LAB population after the 12th day might be due to the disappearance of homofermentative tetrads. In the later phase of the fermentation, the increased acid production might be due to the growth of *L. plantarum* (Karki et al., 1993). Yeast and moulds growths were inhibited by the end products of both homofermentative and heterofermentative LAB which interferes with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH, and inhibiting a variety of metabolic functions (Savard et al., 2002). The lactic acid concentration increased even after the growth ceased on the 9th day of fermentation. This is because the growth of the LAB entered the stationary phase and at

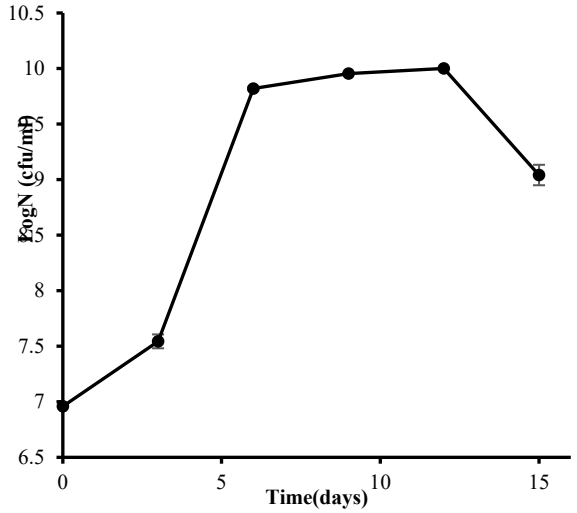

Fig. 2: Changes in the LAB population during the natural fermentation of Rohu pickle.

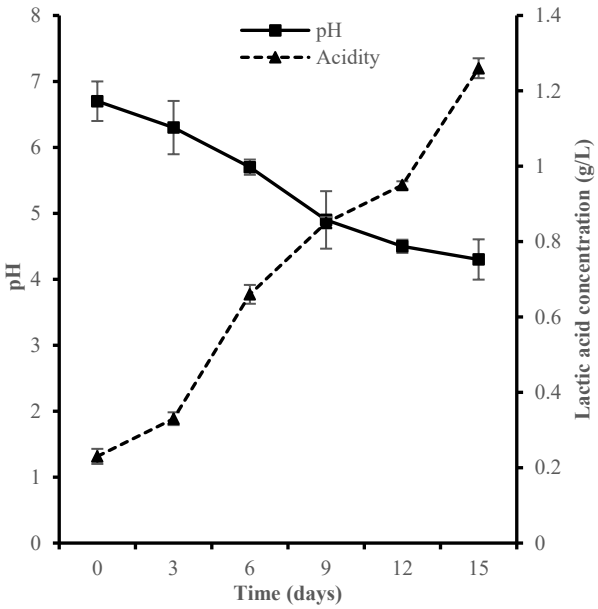

Fig. 3: Chemical changes during natural fermentation of fish pickle.

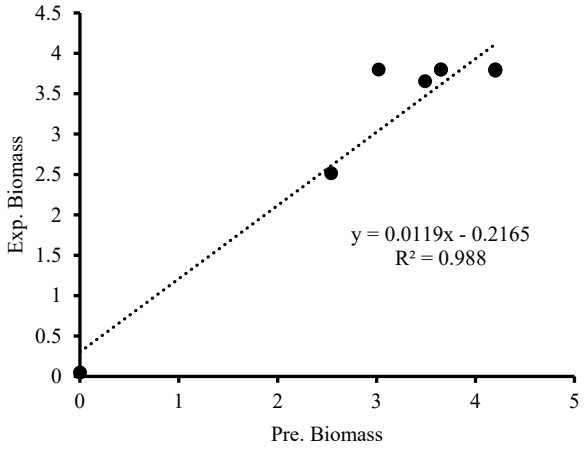

Fig. 4: A plot of predicted and experimental biomass.

this stage probably due to limitation of nutrients other than the carbon source, thereby leading to energy uncoupling of growth and lactic acid production. After 12th day, the lactic acid concentration decreased till the end of fermentation at 15th day which may be due to the limitation of fermentable sugar i.e. carbon source in the substrate (Giraud et al., 1991).

3.2 Acidity and pH

The temperature of fermenting pickles remained around 28–32 °C. The pH of a pickle decreased significantly from 6.5 to 4.2, due to the production of lactic acid by LAB. A significant increase in titratable acidity (as % lactic acid) was observed during fermentation i.e. increased from an initial value of 0.23% to 1.26% at the end of 15th days of fermentation. The chemical change during the natural fermentation of pickle is shown in Fig 3.

The above result complies well with Dai et al. (2013) who concluded that as the fermentation days increased, the acidity increased, while pH decreased till maximum values which was on the 8th day of fermentation. Furthermore, the result was also in accordance with Hossain et al. (2019) who found that the pH of fermentation substrate decreased from 5.53 to 4.35 as the fermentation ended due to the growth of LAB, which converted the fermentable sugars into lactic acid.

3.3 Microbial growth model

Modified Gompertz and Logistic models were used to describe the growth kinetics of LAB in pickles as shown in Table 1. The lag period (λ), the maximum specific growth rate (μ_m), and the log increase in population (A) reached in the culture was estimated and the values of these parameters and the statistical analyses are given in Table 4.

From Table 4, it was observed that the value of R^2 ranges between 0.978 and 0.988 and the lowest χ^2, RMSE, and, RSS values ranging between 0.55 to 0.64, 0.42 to 0.46, and, 0.64 to 0.68, respectively. The value of R^2 obtained for the Gompertz model was not significantly different than the Logistic model and similar values were also obtained for χ^2, RMSE, and RSS. The Logistic model gave the best fit of the data although the results signified that both models are well-fitted with the experimental data. According to this model, the maximum specific rate of the bacteria was completely inhibited at a lactic acid concentration of 0.21 ($\grave{\mu}_{max}$) which was in agreement to the result obtained by Munanga et al. (2016) which shows that all the metabolic activities of *L. plantarum* were completely inhibited at acid growth rate of 0.28.

Variations of experimental and predicted biomass as relative to cell populations with fermentation time are given in Fig. 4. It shows that the biomass predicted by the Logistic model compared with the experimental data which are bounded around the straight-line representing data found by computation. This indicates the good fitting of the mathematical model in describing the growth behaviour of LAB during pickle fermentation where initially, there was no competition for nutrients at which the microbial population growth followed the exponential law.

The Logistic equation considers only biomass concentration while disregarding substrate utilization. However, the logistic equation has also been used by many investigators to describe batch microbial growth (Schmidt et al., 1985; Simkins et al., 1984; Kingsland, 1995).

3.4 Product formation models

The kinetic parameters were evaluated using the equation shown in Table 2. The results of statistical analyses of lactic acid on models applied for fish pickles are given in Table 5.

In all cases, the values of R^2 for the models are greater than the acceptable threshold of 0.90 which indicates a good fit (Guan & Yoa, 2008). It was seen that the value of the coefficient of determination ranges between 0.961 and 0.982 and the χ^2, RMSE and RSS ranging between 0.122 to 0.174, 0.461 to 0.48, and 0.9091 to 0.94, respectively. Based on the results, Luedeking-Piret model fulfilled all the criteria for the goodness of fit describing the lactic acid production behaviour during fish pickle fermentation.

The kinetic parameters versus the growth associated coefficient ‘m’ of 0.819 and non-growth associated coefficient ‘n’ of 0.0059 was obtained by fitting the

Table 5: Statistical results of product formation models.

Model	Parameters	R^2	χ^2	RMSE	RSS
Luedeking-Piret	m = 0.819 n = 0.0059	0.982	0.122	0.461	0.9091
Monteagudo et al.	P_{max} = 116.544 g/L m = 0.1104 n = 0.0042	0.961	0.174	0.480	0.9093
Balannec et al.	$(HL)_{inh}$ = 21.486 g/L m = 0.0348 n = 0.0153	0.976	0.164	0.465	0.940

experimental data in Luedeking-Piret model using the non-linear least-squares which revealed that the kinetics of lactic acid production in fish pickle fermentation was a mixed type (Mavituna & Sinclair, 2008). In other words, it follows both growth-associated product formation kinetics and non-growth associated production formation kinetics or lactic acid was produced during the growth and stationary phases of the microorganisms. Since the value of 'm' is dominating over 'n', the lactic acid production kinetics was more of growth associated.

4.0 Conclusions

Rohu *Fish* was used to prepare fish pickles by natural fermentation which is a traditional method of its preparation. Cell growth and multiplication have been observed to follow the modified Logistic model and the parameters versus the maximum specific growth rate (μ_{max}), lag period (λ), and log increase in population (A) were determined to be 0.21 h^{-1}, 71.7 h and 4.53, respectively. Little substrate inhibition/product inhibition to microbial activity has been observed. The acidity of pickle increased up to 1.26% in 15 days from 0.23% while the pH of the pickle decreased down to 4.2 from 6.5. Lactic acid production was represented by Leudeking-Piret et al. model and based on the estimated kinetic parameter values versus the growth associated coefficient (m) = 0.81 and non-growth associated coefficient (n) = 0.0059. It was noticed that lactic acid production by LAB was mixed type and the growth associated coefficient (m) has been dominating over the nongrowth-associated coefficient (n). The high significance of correlation ($R^2 = 0.98$) and low values of RMSE (0.461), χ^2 (0.122), and RSS (0.9091) were observed with the experimental and predicted results. Thus, it was found that these models could adequately describe the biochemical changes during LAB growth in the fish pickles.

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