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Study on optimization of process parameters for enhancing the multi-hydrolytic enzyme activity in garbage enzyme produced from preconsumer organic waste

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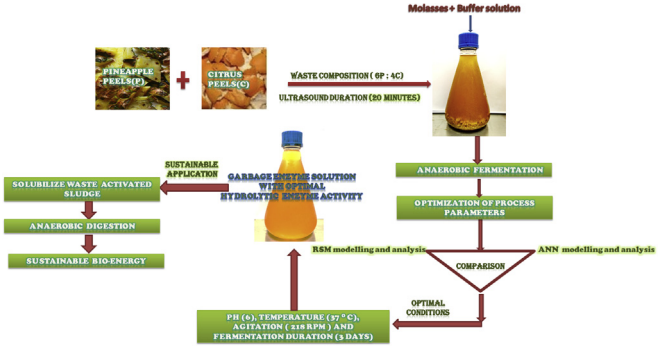
HIGHLIGHTS

- A crude mixed hydrolytic enzyme seems to be a good substitute than single enzyme.
- Garbage enzyme (GE) possesses protease, lipase and amylase activity.
- To produce GE with higher hydrolytic enzyme activity needs optimized parameters.
- Statistical model for GE production were determined.
- GE significantly utilizes for different sustainable environmental applications.

GRAPHICAL ABSTRACT



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ABSTRACT

The garbage enzymes produced from preconsumer organic waste containing multi hydrolytic enzyme activity which helps to solubilize the waste activated sludge. The continuous production of garbage enzyme and its scaling up process need a globe optimized condition. In present study the effect of fruit peel composition and sonication time on enzyme activity were investigated. Garbage enzyme produced from 6 g pineapple peels: 4 g citrus peels pre-treated with ultrasound for 20 min shows higher hydrolytic enzymes activity. Simultaneously statistical optimization tools were used to model garbage enzyme production with higher activity of amylase, lipase and protease. The maximum activity of amylase, lipase and protease were predicted to be 56.409, 44.039, 74.990 U/ml respectively at optimal conditions (pH (6), temperature (37 °C), agitation (218 RPM) and fermentation duration (3 days)). These optimized conditions can be successfully used for large scale production of garbage enzyme with higher hydrolytic enzyme activity.

1. Introduction

In the developing world, enormous amount of fruit as well as vegetables solid waste are generated primarily due to higher production, lack of appropriate preservation and transportation process (Mahmood et al., 1998). Also, these types of organic waste are resulting from peeling and trimming of fruits and vegetable in household kitchens and food industries (Bouallagui et al., 2005) The food based organic waste comprises a major fraction in increasing municipal solid organic waste due to urbanisation and increasing standard of living all over the globe. Ariunbaatar et al. (2014) stated that the generation of food waste will increase up to 44% by 2025, thus management of organic solid waste (OSW) will grow into a major issue all over the globe. OSW containing

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huge amount of organic matter which ultimately degrades to produce carbon dioxide and methane, as the conventional disposal of organic solid waste results in serious environmental pollution and health risks problems to living organisms. From an environmental perspective, there is a crucial need to develop appropriate alternate waste management technology for the utilization of organic wastes as well as to minimize the pollution problems created by them (Anto et al., 2006; Neves et al., 2008; Dhanalakshmi and Alwar, 2012). The chemical complexity, easy degradability, higher moisture content and nutrient rich composition of organic food waste (Kiran et al., 2014), made them as a useful resource for the production of higher value added products such as fuels, chemicals and biochemicals through fermentation process (Chanakya et al., 1999; Sakai et al., 2004; Wang et al., 2005; Zhang et al., 2013; Melikoglu et al., 2013) Globally, the interest of biochemical products (organic acids, enzymes, biopolymers etc.) production from organic waste is increasing day by day (Yuan et al., 2016). Among them, the enzymes play an important role to achieve zero discharge of organic solid waste from different sector by improving biological remediation process to recover valuable resources (Kavitha et al., 2013). The enzymes which are presently used in environmental applications are quiet expensive because of the cost of production and purification. Chamraj Gokul et al. (2011) deciphered that fermentation technology can be applied to effectively utilize vegetable wastes for production of industrially significant enzymes and estimated that protease specific activities for pumpkin peels is 13.44 U mg /protein/ ml, which is comparable higher than using peels of cauliflower and cabbage as raw material. Amin et al. (2014) reported that maximum activity of lipase found after 96 h of reaction in the fermentation medium with initial pH 4 using agricultural waste as raw material.

Recently researchers producing a mixture of crude hydrolytic enzymes through fermentation process seem to be a good substitute and perform better than expensive single enzymes (Enu et al., 2006; Wei et al., 2015). Many researchers suggested that if the crude enzymes activity of biological solution is higher, it can be used directly without any recovery process in a feasible and economical way (Parawira, 2012; Leung et al., 2012; Kiran et al., 2014). Garbage enzyme(GE) was first developed from house hold fruit and vegetable waste and this garbage enzyme functions in the same way as normal enzymes in achieving a higher degree of degradation within a shorter time at suitable environmental conditions (Joean oon, 2008).Thus it helps to promote recycling of organic solid waste back. GE can be utilized as a low-cost alternative to improve wastewater treatment processes by removing the impurities and bacteria (Khairul and shamila, 2012; Bhavani prakash, 2012; Fazna nazim and Meera, 2013).

The analysis of the above cited literature concludes that garbage enzyme was used only for mainly for wastewater treatment and no studies were reported on usage of garbage enzyme for organic solid waste treatment. In our earlier works, garbage enzyme produced by the fermentation of different preconsumer organic waste was tested for bio catalytic activity and its effects on solubilization of waste activated sludge (WAS) were reported (Arun and Sivashanmugam, 2015). The result confirms that garbage enzyme produced from pineapple and citrus peels possesses maximum protease, lipase and amylase activity, which help to increase the solubilisation of TKN(Total Kjeldhal Nitrogen), COD (Chemical oxygen demand), and TP (Total phosphorus) nearly 15–20%, 20–25%, 9–11% respectively in waste activated sludge.

The production of garbage enzyme with higher activity of lipase, amylase and protease is needed to cater for treatment of larger quantity of industrial waste activated sludge generated. This necessitates the optimizing of various parameters to improve the activity of crude enzyme mixtures to reduce their cost and cost of application. Till date no study was reported to optimize the parameters responsible for enhancing the activity of multi-enzyme in garbage enzymes solution produced from organic solid waste.

The statistical methods for optimization are gaining an increasing importance, as this methodology can save time and cost (Vitcosque et al., 2012). Currently, Response surface methodology (RSM) and Artificial neural networks (ANN) are potent mathematical methods applicable for modelling and simulation of various processes parameters in many real applications. RSM and ANN have been commonly applied for the modelling and optimization of many biochemical processes used for the production of primary metabolites and secondary metabolites (Dasu and panda, 2002; Dahunsi et al., 2016). Generally in the mathematical methods, the models are initially developed according to the functional relationships between input parameters and response (output) of the process using experimental data. Subsequently, the developed models are used to estimate the optimal values of input parameters to maximize or minimize the response (Emeko et al., 2015).

To achieve large scale production of garbage enzyme with enhanced multi- hydrolytic enzyme activity, the fermentation process parametric conditions have to be optimized and are reported in the present work. Initially the effect of fruit peel (pineapple and citrus) composition on enzyme activity of garbage enzyme was studied. The determination of ammonium sulphate maximum and minimum saturation cut off value for partial purification process for lipase, amylase and protease in garbage enzyme solution was studied. The effect of sonication time on enzyme activity of garbage enzyme was investigated to obtain the optimal duration of ultrasound required to pre-treat fruit waste to produce garbage enzyme solution with higher activity of all the hydrolytic enzymes. Subsequently, the RSM and ANN optimization methodology have been conducted and compared to determine the optimal factors (pH, temperature, and agitation and fermentation time) for producing a garbage enzyme with higher activity of lipase, amylase and protease.

2. Materials and method

2.1. Production of garbage enzyme

Pineapple and citrus fruit dregs from fruit shop were collected and mixed equally. From this mixture 90 g was taken in air tight container and 30 g of molasses and 300 ml of water were added. The container was placed in a well-ventilated, dry and cool area for fermentation for three months. After three months the garbage enzyme solution was filtered and centrifuged at 5000 rpm for 30 min. The obtained supernatant was used for further investigation.

2.2. Optimization of partial purification process

In present study ammonium sulphate precipitation methodology is used for partial purification of amylase, protease and lipase in garbage enzyme solution obtained from preconsumer organic waste. The garbage enzyme was taken in a beaker, sufficient quantity amount of ammonium sulphate was added to make the solution to 10 % saturated and stirred for 30 min. This 10% saturated solution was centrifuged at 5000 rpm for 30 min. The pellet (P) was re-suspended in a beaker with the minimal volume of homogenization buffer and labelled as 10% P and kept in refrigerator. The 10% saturated supernatant was taken in another beaker, enough quantity of ammonium sulphate was added to make the solution to 20% saturated and stirred for 30 min. This 20% saturated solution was centrifuged at 5000 rpm for 30 min. The pellet (P) was re-suspended in a beaker with the minimal volume of

homogenization buffer and labelled as 20% P and kept in refrigerator. The same procedure was continued to produce 30%P, 35%P, 40%P, 45%P, 50%P, 55%P, 60%P, 65%P, 70%P, 75%P, 80%P and 85%P.

The Lowry assays was performed to find out the total protein concentration (Lowry et al., 1951). Amylase activity for each of the above fractions was determined using Ezeji and Bahl methodology (Ezeji and Bahl, 2006) and the data was recorded at 540 nm. Proteolytic activity measurement (Tsuchida et al., 1986) was used to determine the enzyme activity for each of these fractions and the absorption data was recorded at 620 nm. Lipase activity was assayed spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate at 410 nm according to the method of Pandey et al. (1999) for each of the above fractions. Graph was plotted between ammonium sulphate saturation Vs total protein and enzyme activity to determine maximum and minimum cut off value for amylase, protease and lipase. Thus, the optimal cut off values of ammonium sulphate saturation for amylase, lipase and protease in crude homogenate was found.

After determining the ammonium sulphate saturation maximum and minimum cut off values, the crude garbage enzyme homogenate obtained was taken in a beaker. The above solution was saturated with minimum cut off value and stirred for 15 min. Then the saturated solution was centrifuged at 6000 rpm for 30 min. The supernatant was taken in another beaker, the solution was saturated to maximum cut off value and stirred well for 15 min. This saturated solution was centrifuged at 6000 rpm for 30 min. The precipitate (Pellet – P) was taken and re-suspended with homogenization buffer in a beaker which was used as partially purified enzyme solution for further studies.

2.3. Determination of enzyme kinetics

Enzyme kinetics studies were used to determine kinetic parametres to get information on enzyme mechanism and their interaction towards substrate. The enzyme is characterized by two constants namely V_{max}, the maximum rate attainable and it is the rate at which the total enzyme concentration is present as the enzyme–substrate complex and K_m, the dissociation constant of the enzyme–substrate complex, [ES].The larger the value K_m, the weaker the binding between enzyme and substrate.

The partially purified enzyme solution was taken and the same was used to determine kinetic parameters using the Line weaver-Burk plot. Protease kinetic parameter was determined by incubating the reaction mixture containing casein with various concentrations (0.2 to 1 mM) at 37 °C for 15 min at pH 6.5 and the reaction was stopped with the addition of 3 ml of 5% trichloroacetic acid. An appropriate blank solution was also prepared and absorbance values at 620 nm were measured with reference to the blank to get the value for reaction velocity. The lipase enzyme kinetics was studied using p-nitrophenyl palmitate (p-NPP) (0.2 – 1 mM) and the reaction rate was assayed for 2 min at pH 8.0 by using spectrophotometric method. The absorbance values obtained was correlated with the amount of product formed and reaction velocity values were obtained. The amylase enzyme kinetic parameters were determined by measuring the initial velocity using various starch concentrations (0.2–1 mM) with the reported methodology (Metin et al., 2010). K_m and V_{max} were determined using the Line weaver-Burk plot.

2.4. Effect of fruit peels composition on enzyme activity of garbage enzyme

Pineapple (P) and Citrus (C) fruit dregs from fruit shop were taken. 1 g of Pineapple waste and 9 g of Citrus waste were mixed to obtain a ratio 1(P):9(C). From this mixture 3 part of waste were taken in air tight container, 1 part of molasses and 10 part of water were added. The container was placed in a well-ventilated, dry and cool area for fermentation for 5 days. After 5 days the garbage enzyme solution was filtered and centrifuged at 5000 rpm for 30 min. The obtained supernatant was used to determine protease, amylase and lipase activity in garbage enzyme solution. The same methodology was followed to obtain the biocatalyst activity of garbage enzyme prepared from different ratio of waste (i.e.) 2(P):8(C), 3(P):7(C), 4(P):6(C), 5(P):5(C), 6(P):4(C), 7(P):3(C), 8(P):2(C), 9(P):1(C).

2.5. Effect of sonication time on enzyme activity of garbage enzyme

The mixture of fruit waste (6P:4C) and phosphate buffer solution (pH 7) at a weight ratio of 3:1 were taken in glass flasks and these were subjected to ultrasound pre-treatment using a sonicator (Model: Lark Classic Model, Power: 45%, frequency: 20 kHz, sonication temperature: 30 °C) with different sonication time of 10, 15, 20, 25, 30, 35, 40, 45, and 50. The control samples were not subjected to the sonication. After the ultrasound pre-treatment, pre-treated fruit waste solution were taken in air tight containers and were named as 5 M, 10 M, 15 M, 20 M, 25 M, 30 M, 35 M, 40 M, 45 M, 50 M according to the duration of ultrasound treatment. To each container molasses and water were added and kept in a well-ventilated, dry and cool area for fermentation for 5 days. After 5 days the garbage enzyme solution was filtered and centrifuged at 10,000 rpm for 30 min. The obtained supernatant was used to determine protease, amylase and lipase activity in garbage enzyme solution. All sonication tests were conducted in triplicate.

2.6. Optimization of process parameters

2.6.1. RSM modelling

Manohar and Divakar (2005) stated that RSM (Response surface methodology) is an empirical modelling system employed for developing, improving, and optimizing complex production processes. In the present study a three-level, four-factor central composite experimental design (CCD) was used. A: pH, B: Temperature, C: Agitation, D: Fermentation time was taken as the input variables; the factor levels were coded as −1 to +1 as presented in Tables 1 and 2. The input variables (factors) and their levels were selected based on preliminary experiments carried out in the laboratory (Li et al., 2012; Chakraborty and Sahu, 2014). According to the CCD, experiments were performed in order to find out the optimum combination and to study the effect of process parameters on multiple enzyme activities i.e. protease, lipase and amylase activities in the garbage enzyme solution. The experimental data obtained from CCD were used to analyse the regression (Design Expert ™ 9.0) and fitted to a second-order polynomial model in order to identify all possible interactions between the factors and is given as

$$Y = a_0 + \sum_{s=1}^{n} a_s x_s + \sum_{s=1}^{n} a_{ss} x_s^2 + \sum_{s=1}^{n} \sum_{s=t}^{n} a_{st} x_s x_t + Z \quad (1)$$

where Y is response (Enzyme activity U/ml), a_0, a_s, a_{ss} and a_{st} are the regression coefficients obtained for constant, linear, quadratic and

Table 1
Experimental ranges and levels of the independent variables for RSM study.

Factors	Units	−1 levels	0 levels	1 levels
pH	–	3	6	9
Temperature	°C	25	35	45
Agitation	RPM	100	200	300
Fermentation time	Days	1	3	5

Table 2
Central Composite Design of four independent variables along with experimental and predicted response.

Run	Factors				Amylase Activity(U/ml)			Lipase Activity(U/ml)			Protease Activity(U/ml)		
	A	B	C	D	E	PR	PN	E	PR	PN	E	PR	PE
1	1	1	−1	−1	14.04	11.73	14.45	10.56	10.71	10.56	26.57	22.860	26.93
2	−1	0	0	0	25.59	29.15	25.51	23.54	26.21	23.54	41.35	38.257	40.06
3	0	0	0	0	55.09	56.19	54.13	40.98	43.57	40.58	72.09	72.678	73.08
4	−1	1	−1	1	11.28	11.55	11.39	9.9	9.36	9.9	16.14	15.680	17.60
5	1	0	0	0	34.64	37.02	35.76	25.96	28.95	25.86	40.65	49.906	40.60
6	0	0	1	0	50.77	52.71	50.89	31.29	35.61	31.29	67.77	71.756	66.76
7	1	−1	−1	−1	6.59	7.61	6.607	5.2	5.21	5.2	10.95	11.630	14.06
8	0	0	−1	0	44.89	48.89	44.81	31.67	33	31.67	58.23	60.407	57.71
9	−1	−1	−1	1	10.47	9.42	10.49	8.02	8.54	8.02	9.08	10.585	9.01
10	−1	−1	−1	−1	6.38	4.69	6.455	5.09	4.91	5.06	8.79	9.446	8.89
11	1	1	−1	1	17.56	17.55	17.75	13.24	13.42	13.24	31.79	34.759	31.21
12	0	0	0	0	59.06	56.19	59.13	49.45	43.57	49.28	76.53	72.678	76.08
13	0	−1	0	0	47.89	51.11	47.75	32.34	35.32	31.60	54.93	58.382	55.00
14	1	−1	1	1	20.93	21.1	20.87	12.09	11.56	11.86	30.39	32.853	29.89
15	0	0	0	0	55.09	56.19	55.13	47.99	43.57	46.28	77.58	72.678	76.08
16	−1	1	1	1	9.48	8.81	9.56	14.32	13.39	15.92	25.69	27.769	24.63
17	1	−1	−1	1	14.17	13.77	14.09	9.65	8.56	9.51	24.94	20.824	23.81
18	0	0	0	−1	38.69	43.27	38.86	36.73	39.03	36.73	59.09	64.147	59.05
19	−1	−1	1	1	9.45	9.93	9.24	11.08	10.43	11.08	21.17	20.579	21.51
20	−1	1	1	−1	8.89	7.46	8.11	7.97	8.56	7.97	22.69	22.505	22.96
21	1	1	1	1	21.78	21.64	21.77	18.9	18.58	18.8	53.84	48.883	52.39
22	1	1	1	−1	17.45	18.85	17.80	15.47	14.04	15.47	34.31	35.564	34.21
23	0	0	0	0	61.55	56.19	61.13	42.11	43.57	42.08	79.05	72.678	79.08
24	0	0	0	0	62.07	56.19	61.13	48.79	43.57	48.58	73.08	72.678	73.08
25	0	0	0	1	45.67	47.03	46.00	39.76	43.12	39.76	70.27	71.376	69.38
26	−1	1	−1	−1	6.98	7.16	6.55	6.74	6.36	6.74	11.54	11.836	11.53
27	1	−1	1	−1	20.07	17.97	19.38	6.36	6.4	6.36	26.08	22.239	26.32
28	0	0	0	0	62.1	56.19	61.13	49.06	43.57	49.08	76.23	72.678	76.08
29	−1	−1	1	−1	7.87	8.23	7.899	6.06	4.97	7.095	18.23	18.020	20.13
30	0	1	0	0	49.89	52.61	49.84	36.87	39.55	36.87	64.88	67.592	63.41

A: pH, B: Temperature, C: Agitation, D: Fermentation time, E: Experimental Values, PR: Predicted Values RSM, PN: Predicted values ANN.

interaction terms, respectively, a is the regression coefficient, n is the number of factors studied and optimized in the experiment, Z is random error. x_s and x_t are independent variables, and s and t are the linear and quadratic coefficients, respectively.

2.6.2. ANN modelling

Artificial neural networks (ANNs) are mathematical models that estimate the function of biological neural networks. In this study, Neural Network Toolbox in MATLAB (R2011a) mathematical software was used for predicting the enzyme activities in garbage enzyme solution. From Table 2, design of experiments (DoE) and the corresponding experimental response were taken for training the network. A three-layer feed-forward neural network (input, hidden and output) methodology with tangent sigmoid transfer function (tansig, Eq. (1)) at hidden layer and a linear transfer function (purelin,Eq. (2)) at output layer were used for training the network. In the present study one input layer with four input variables (pH, temperature, agitation, and fermentation time), hidden layers (10 neurons) and one output layer with one output variable (Fig. S1) were taken. Marquardt-Levenberg (ML) back-propagation algorithm (nonlinear least-squares algorithm) was applied to the network training with 60% of the data for the training set, 20% for the validation set and another 20% of the data for the test set. Algorithms [35]of tansig and purelin are as follows:

$$tansig(x) = \frac{2}{1+e^{-2x}} - 1 \tag{2}$$

$$purelin(x) = x \tag{3}$$

2.6.3. Performance of RSM and ANN modelling

To determine the fitting and prediction accuracy of RSM and ANN model, both the model were compared for the design of experiment with which they were trained. The comparison was made according to the statistical error analysing formulas and are given as follows.

$$\text{Correlation coefficient}(R^2) = 1 - \frac{\sum_{j=1}^{n}(Y_{j,p} - Y_{j,e})^2}{\sum_{j=1}^{n}(Y_{j,p} - Y_e)^2} \tag{4}$$

$$\text{Root mean square error } (RMSE) = \sqrt{\frac{\sum_{j=1}^{n}(Y_{j,e} - Y_{j,p})^2}{n}} \tag{5}$$

$$\text{Standard error of prediction } (SEP) = \frac{RMSE}{Y_e} * 100 \tag{6}$$

$$\text{Absolute average deviation } (AAD) = \frac{100}{n}\sum_{j=1}^{n}\frac{|(Y_{j,p} - Y_{j,e})|}{|(Y_{j,e})|} \tag{7}$$

where $Y_{j,e}$ is the experimental data, $Y_{j,p}$ is the corresponding predicted data, Y_e is the mean value of experimental data and n is the number of the experimental data.

In addition, modelling abilities of the RSM and ANN models for optimizing the process parameters to enhance co-production of amylase, lipase and protease in garbage enzyme solution were determined by plotting a graph between the predicted values for amylase, lipase and protease activity against the corresponding experimental values.

3. Result and discussion

3.1. Partial purification-ammonium sulphate precipitation

Purification of amylase, lipase, and protease from crude garbage enzyme homogenate were performed using ammonium sulphate

precipitation process. To determine the optimal ammonium sulphate saturation cut off values a graph was constructed between ammonium sulphate saturation percent vs. total protein as well as enzyme activity and is presented in Fig. 1. From the Fig. 1 the maximum and minimum ammonium sulphate saturation cut off value for amylase, lipase and protease were found to be 35% & 65%, 40% & 75% and 20% & 60% respectively. Between these ranges desired enzyme was precipitated out from the crude solution. Similar partial purification methodology was reported by Ilaria et al. (2014), Amid et al. (2014), Fernando de Almeida et al. (2016) for the purification of amylase, protease and lipase produced from organic waste materials respectively.

3.2. Determination of enzyme kinetic parameters

In general the enzyme kinetic parameters K_m and V_{max} of enzymes depend on the substrate used and the reaction conditions. In present research work, the V_{max} and K_m of amylase, protease and lipase in garbage enzyme were derived from the Lineweaver Burke plot and the result obtained is presented in Fig. S2a–c respectively. From Fig. S2a it is observed that the amylase in garbage enzyme solution has K_m of 0.4015 mM and V_{max} of 15.8018 μmol/min/mg for starch. Fig. S2b illustrate that the protease in garbage enzyme has K_m of 0.5568 mM and V_{max} of 18.286 μmol/min/mg for casein. It is observed from the Fig. S2c that lipase in garbage enzyme solution has the K_m of 0.333 mM and V_{max} of 12.625 μmol/min/mg for pNPP at pH 7. From Fig. S2b and S2c, it observed that the low values of K_m indicate high affinity of the protease and lipase enzyme towards the casein and pNPP respectively. Hamilton et al. (1999) reported that the low values of K_m indicate high affinity of the enzyme towards the substrate and it helps to maximize the rate of reaction. Accordingly these observations confirm that amylase, lipase and protease in garbage enzyme need low substrate concentration to achieve its maximum catalytic efficiency, as they have a small value of K_m.

3.3. Effect of fruit peels composition on enzyme activity of garbage enzyme

In the present study cell free amylase, lipase and protease activities in garbage enzyme solution produced from different composition of pineapple and citrus waste were determined. The observed result is presented in Fig. 2 and it reveals that all three hydrolytic activities increase gradually reaching a maximum for the ratio of 6 g of pineapple fruit waste to 4 g of citrus fruit waste and decrease gradually from the ratio of 7 g of pineapple fruit waste to 3 g of citrus fruit waste. This observation confirms that the ratio of waste composition has a considerable effect on hydrolytic activity of garbage enzyme solution.

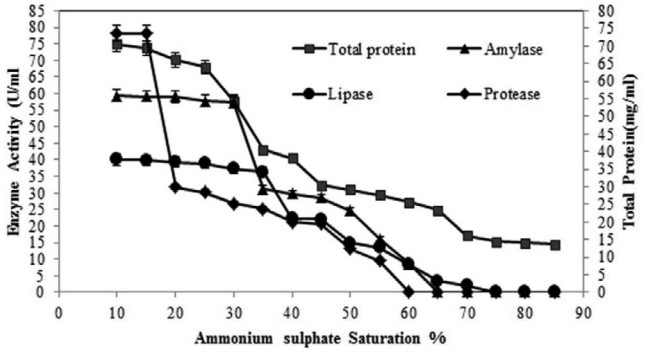

Fig. 1. Optimization of partial purification process of hydrolytic enzymes in garbage enzyme solution.

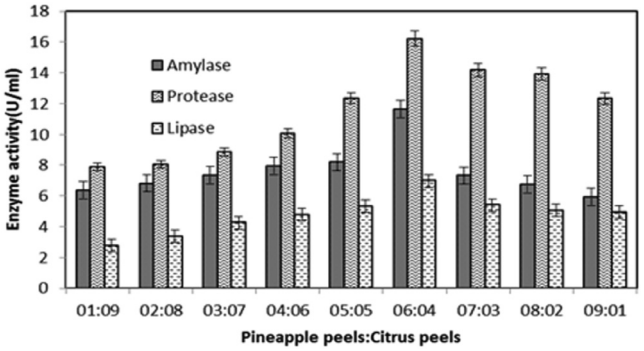

Fig. 2. Effect of fruit peels on hydrolytic enzyme activity of garbage enzyme.

3.4. Effect of sonication time on enzyme activity of garbage enzyme

Yu et al. (2007) reported that low frequency ultrasound could enhance the enzyme activities in the organic solid waste and suggested that ultrasound is a good method to extract enzymes from organic solid waste. In the present study, sonication treatment was performed to determine the optimal duration of ultrasound required to pre-treat fruit waste (6P:4C) in buffer solution to produce garbage enzyme solution with higher activity of hydrolytic enzymes and the observed result is presented in Fig. 3. From the Fig. 3, it is observed that all three hydrolytic activities in garbage enzyme solution increase gradually from 5 min to 15 min and then significantly achieves the maximum enzyme activity at 20 min due to the application of ultrasound, which helps to disintegrate the cell wall of organic waste to extract extracellular enzyme and some intercellular enzyme from the organic waste materials. After 20 min the enzyme activities start decreasing gradually up to 50 min. Similarly Wang et al. (2013) reported that prolonged exposure of ultrasound generate mechanical shear which may affect the activity of biomolecules. Thus, the optimum time duration for sonication is 20 min and it is adequate for disintegrating the fruit waste (6P:4C) to produce garbage enzyme solution with higher activity of amylase, lipase and protease.

3.5. Optimization of multi-hydrolytic enzyme activity in garbage enzyme solution

3.5.1. Response surface methodology modelling analysis

The efficient and optimal co-production of hydrolytic enzyme mixture (lipase, amylase and protease) in garbage enzyme solution is dependent on various parameters such as pH (A), temperature (B), agitation (C) and fermentation time (D). Optimization was carried out using DOE software, to obtain the best environment

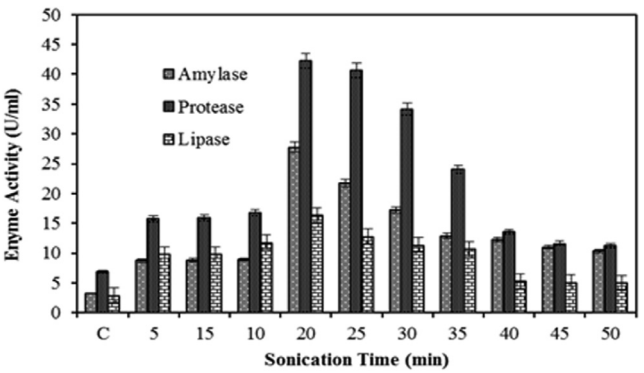

Fig. 3. Effect of sonication time on hydrolytic enzyme activity of garbage enzyme.

Table 3
Analysis of Variance (ANOVA) for the fitted polynomial quadratic model of Hydrolytic enzyme in garbage enzyme solution.

Source	Sum of Squares			df			Mean Square			F Value			p-value Prob > F					
	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3	E1	E2	E3
Model	11892.97	6986.28	16823.43	14	14	14	849.50	499.02	1201.67	61.36	38.04	51.62	<0.0001	<0.0001	<0.0001	S	S	S
A	278.79	33.92	610.63	1	1	1	278.79	33.92	610.63	20.14	2.59	26.23	0.0004	0.0286	0.0001			
B	10.17	80.56	381.71	1	1	1	10.17	80.56	381.71	0.73	6.14	16.40	0.4049	0.0256	0.0010			
C	65.47	30.60	579.59	1	1	1	65.47	30.60	579.59	4.73	2.33	24.90	0.0461	0.1475	0.0002			
D	63.58	75.15	235.16	1	1	1	63.58	75.15	235.16	4.59	5.73	10.10	0.0489	0.0302	0.0062			
AB	2.73	16.38	78.15	1	1	1	2.73	16.38	78.15	0.20	1.25	3.36	0.6633	0.2813	0.0869			
AC	46.55	1.26	4.14	1	1	1	46.55	1.26	4.14	3.36	0.096	0.18	0.0866	0.7609	0.6792			
AD	2.05	0.086	64.88	1	1	1	2.05	0.086	64.88	0.15	6.523E−003	2.79	0.7056	0.9367	0.1158			
BC	10.51	4.61	4.39	1	1	1	10.51	4.61	4.39	0.76	0.35	0.19	0.3972	0.5620	0.6703			
BD	0.12	0.39	7.32	1	1	1	0.12	0.39	7.32	8.473E−003	0.030	0.31	0.9279	0.8648	0.5833			
CD	9.20	3.34	2.02	1	1	1	9.20	3.34	2.02	0.66	0.25	0.087	0.4278	0.6212	0.7726			
A^2	1382.61	662.47	2118.74	1	1	1	1382.61	662.47	2118.74	99.87	50.51	91.01	<0.0001	<0.0001	<0.0001			
B^2	48.48	97.53	243.35	1	1	1	48.48	97.53	243.35	3.50	7.44	10.45	0.0809	0.0156	0.0056			
C^2	75.15	222.18	112.74	1	1	1	75.15	222.18	112.74	5.43	16.94	4.84	0.0342	0.0009	0.0438			
D^2	315.53	16.13	62.63	1	1	1	315.53	16.13	62.63	22.79	1.23	2.69	0.0002	0.2849	0.1218			
Residual	207.66	196.75	349.21	15	15	15	13.84	13.12	23.28									
Lack of Fit	151.69	124.35	313.61	10	10	10	15.17	12.44	31.36	1.36	0.86	4.40	0.3879	0.6098	0.0577	NS	NS	NS
Pure Error	55.96	72.40	35.60	5	5	5	11.19	14.48	7.12									
Cor Total	12100.63	7183.03	17172.64	29	29	29												
R-Squared	0.9828	0.9726	0.9797															
Adj R-Squared	0.9668	0.9470	0.9607															
Pred R-Squared	0.9503	0.9345	0.9105															
C.V. %	12.45	15.58	11.27															
Std. Dev.	3.72	3.62	4.83															

A: pH, B: Temperature, C: Agitation, D: Fermentation time, E1: Amylase, E2: Lipase, E3:Protease, S- Significant,NS- Non Significant.

conditions for the co-production of lipase, amylase and protease enzymes mixture and the activities of these enzymes were used as a response. The maximum (+1) and minimum (−1) levels of parameters (A, B, C, D) taken for the test in the CCD are presented in Table 1. To enhance the accuracy of the regression model, the centre point (0) was replicated six times. A total of 30 experiments were performed according to the experimental design produced by DOE software (Table 2). All the experiments were conducted in triplicate and average enzyme activity obtained was taken as response.

The experimental results obtained according CCD experimental design was fitted with the second-order polynomial equation. The final quadratic model equation obtained in terms of coded factors (pH (A), temperature (B), agitation (C) and fermentation time (D)) is as follows:

$$\begin{aligned}\text{Amylase (U/ml)} = {} & 56.19 + 3.94\ A + 0.75\ B + 1.91C \\ & + 1.88\ D + 0.41\ AB + 1.71\ AC + 0.36\ AD \\ & - 0.81\ BC - 0.086\ BD - 0.76\ CD \\ & - 23.10\ A^2 - 4.33\ B^2 - 5.39\ C^2 \\ & - 11.04\ D^2 \end{aligned} \quad (8)$$

$$\begin{aligned}\text{Lipase (U/ml)} = {} & 43.57 + 1.37\ A + 2.12\ B + 1.30\ C + 2.04\ D \\ & + 1.01\ AB + 0.28\ AC - 0.073AD + 0.54\ BC \\ & - 0.16BD + 0.46CD - 15.99A^2 - 6.14\ B^2 \\ & - 9.26\ C^2 - 2.50\ D^2 \end{aligned} \quad (9)$$

$$\begin{aligned}\text{Protease (U/ml)} = {} & 72.68 + 5.82\ A + 4.60\ B + 5.67\ C \\ & + 3.61\ D + 2.21\ AB + 0.51\ AC \\ & + 2.01\ AD + 0.52\ BC + 0.68BD \\ & + 0.36CD - 28.60A^2 - 9.69\ B^2 \\ & - 6.60\ C^2 - 4.92\ D^2 \end{aligned} \quad (10)$$

The statistical significance and fitness of the quadratic polynomial model equation were confirmed by F test and analysis of variance (ANOVA), which is presented in Table 3. Li et al. (2013) reported that the determination of regression coefficients (R^2) provides a measure of variability in the observed response values and the same were determined by the experimental factors and their interactions. In this present study the R^2 value for amylase, lipase and protease were found to be 0.9828, 0.9726, and 0.9797 respectively, indicating model to be more appropriate and could be used for quantitative prediction of hydrolytic enzymes activity in garbage enzyme solution. From Table 3, it is observed that the model F-value for amylase, lipase and protease are 61.36, 38.04, 51.62 respectively as well as the high value of adj-R^2 0.9668, 0.9470, 0.9607 for amylase, lipase and protease implying that the model was significant. Also, the lack of fit test result of amylase, lipase and protease were 1.36, 0.86, and 4.40 respectively indicating that model was insignificant relative to pure error. The coefficient of variance percentage (CV %) values of amylase, lipase and protease are 12.45, 15.58, and 11.27 respectively and these smaller values signify that the aberrations between predicted and experimental values were low. The statistical results presented in Table 3 reveal that there is a good agreement between the experimental and pre-

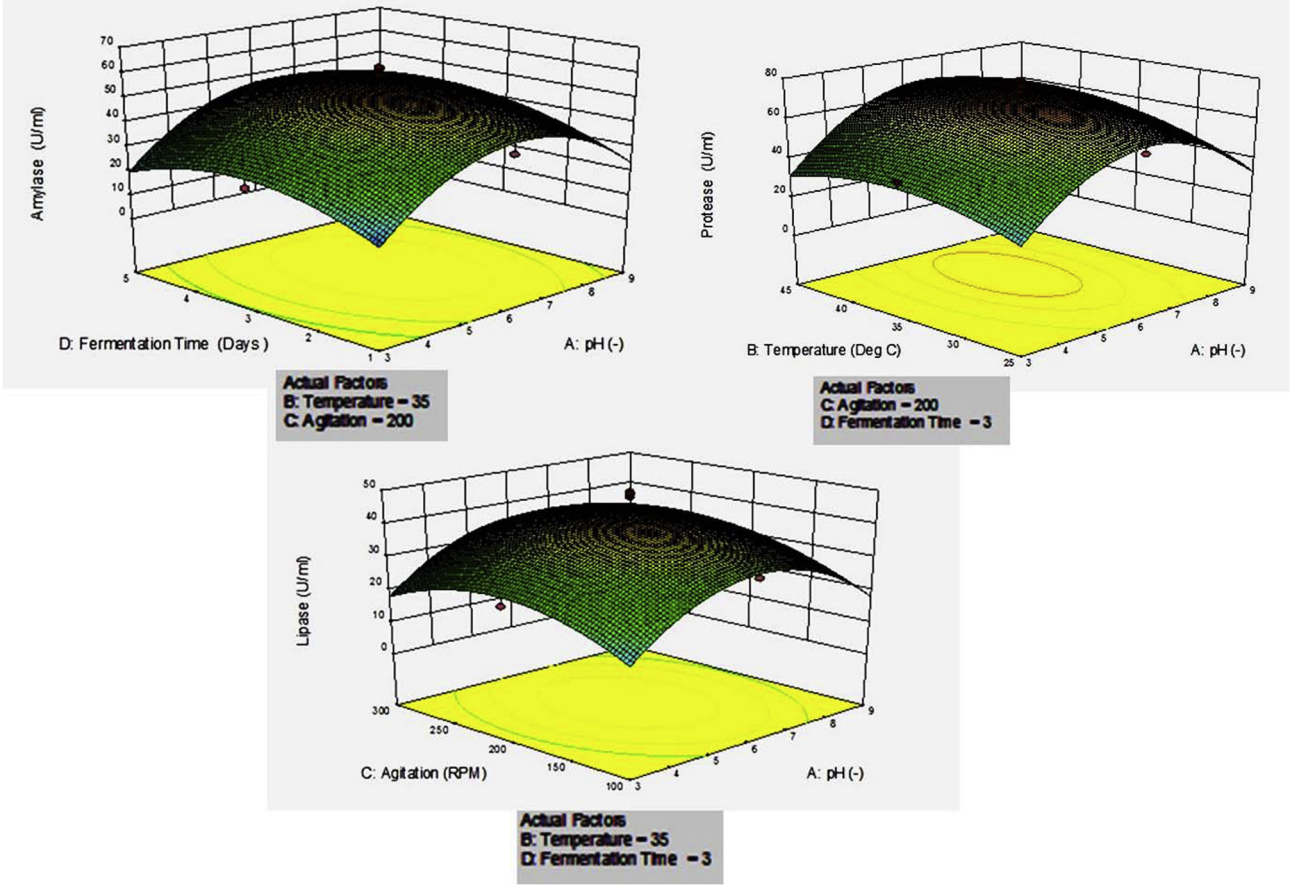

Fig. 4. Three-dimensional response surfaces plots showing the significant interaction effect of four independent variables on the three responses.

dicted values and the determined mathematical models were suitable for the simulation of co-production of amylase, lipase and protease.

The significance of each coefficient was determined by analysing the P-values and these are also required to understand the pattern of mutual interaction between the independent variables. The smaller the magnitude of the P, the more significant is the corresponding co-efficient (Debabrata et al., 2013). In the present study the regression and P value analysis indicates that all the primary four factors A, B, C, and D had a significant influence on amylase, lipase, and protease activity in garbage enzyme solution.

The contour 3d surface plots were constructed by plotting the responses (enzyme activity) on z axis versus the two independent variables, while other variables were at their optimal levels. Mehri et al. (2013) stated that the circular (insignificant) or elliptical (significant) nature of response surface contours around the stationary point can be used to determine the main interaction effects between the experimental variables. Fig. 4 presents the significant interaction effect of four independent variables on the three responses (amylase, lipase and protease activity). From the Fig. 4, it is observed that the interaction between fermentation time with pH, agitation with pH and temperature with pH were significant and a small change of these variables causes a big change on amylase, lipase and protease activity in garbage enzyme solution.

The numerical global optimized conditions for co-production of amylase, lipase and protease in garbage enzyme solution were found to be pH (6), temperature (37 °C), agitation (218 RPM) and fermentation duration (3 days). The predicted maximum amylase, lipase and protease activity in garbage enzyme at optimized conditions were 56.409, 44.039, 74.990 U/ml respectively with desirability value of 0.906. The verification of this predicted optimal condition was conducted in triplicate and the maximum amylase, lipase and protease activity in garbage enzyme solution were found to be 57.289, 43.987, 76.024 U/ml respectively and these were very close to the predicted values. Results suggested that the optimal conditions achieved had the least error and can be essentially applied to produce garbage enzyme solution with higher activity of amylase, lipase and protease.

3.6. Neural network modelling analysis

Chakraborty and Sahu (2014) reported that ANN model development for a system should require statistically significant small data in the input and output domains like RSM model. As a result the experimental data obtained through RSM (Table 2), could be sufficient to build efficient ANN model. In this study, Neural Network Toolbox in MATLAB (R2011a) mathematical software was used for predicting the hydrolytic enzyme activities in garbage enzyme solution. The ANN used in this study provided with weights are listed in Table 4. Fig. 5a, b, and c present the scatter diagrams that compare the experimental data versus the computed neural network data in training, validation, test and all prediction set for Amylase, lipase and protease respectively.

From the Fig. 5a, it is observed that the NN (neural network) for Amylase model confirms that R values as 0.99899, 0.99495, 0.99643, 0.99686 for training, validation, test and all prediction set respectively and these were significant. Similar behaviour is observed for lipase NN model (Fig. 5b), and protease NN model (Fig. 5c) indicating the significance of R values for training, validation, test and all prediction set. The result obtained by ANN model trained using RSM experimental data illustrate that R values between experimental response and ANN predicted response in all the cases were precise in predicting the amylase, lipase and protease activity in garbage enzyme solution. Consequently, all the variables studied in this present work were significant and cannot be neglected. Thus, this experiment confirms the applicability and

Table 4
Weights and Bias of the Neural Network.

	W_1												Bias(b1)			W_2		
	Variables															N	Weights	
N	pH			Temperature			Agitation			Fermentation time								
	A	L	P	A	L	P	A	L	P	A	L	P	A	L	P		A	L	P
1	−0.042	−0.508	0.451	−1.230	1.645	1.78	−2.489	0.498	1.6883	1.150	−1.194	0.016	1.817	3.126	2.623	1	−0.490	−0.975	1.352
2	0.028	1.012	1.191	−1.652	−1.494	−3.036	2.353	−2.275	−1.561	−0.813	−0.003	1.554	1.784	−1.786	−1.178	2	−1.448	−0.684	−0.857
3	−0.243	0.751	1.857	−1.707	1.308	0.172	0.197	1.999	1.587	−1.809	1.229	−0.397	1.262	−1.464	−1.408	3	0.239	−1.124	−0.502
4	−1.754	−2.556	−1.157	0.023	1.138	1.807	1.419	0.501	0.170	−0.579	0.482	−0.472	1.627	2.294	1.170	4	1.085	1.140	−0.886
5	−0.798	−1.345	−0.724	−1.765	1.039	1.395	−0.367	−0.204	0.909	−1.549	1.094	1.62	0.507	0.886	1.844	5	0.931	−0.083	0.559
6	0.186	−0.290	−1.122	2.818	2.075	3.584	0.380	−0.953	−0.077	−0.092	−0.149	0.500	0.458	−2.676	1.153	6	0.817	−0.817	0.709
7	1.595	1.013	3.531	0.348	0.874	0.054	−1.299	−0.203	−0.212	1.556	1.980	0.366	1.264	1.821	1.109	7	1.871	1.237	1.195
8	−1.078	−0.941	−0.935	1.988	−1.287	−2.486	0.886	−1.299	−1.305	0.694	1.146	−0.134	−1.159	−1.438	−1.154	8	0.298	−0.693	0.666
9	0.079	0.409	−1.554	2.880	−0.295	−2.147	0.321	0.916	1.530	0.340	−1.278	0.312	−1.821	4.132	2.062	9	−1.031	−0.357	0.754
10	−0.975	−2.755	−1.547	−0.015	0.946	−1.280	−2.558	−0.376	0.046	−0.681	−0.074	0.773	−2.405	−2.062	−2.968	10	−2.270	−0.629	0.380
															Bias(b2)		−3.201	−2.983	−2.203

N-Neurons, A-Amylase, P-Protease, L-Lipase, Matrix of weights, W1: weights between input and hidden layers; W2: weights between hidden and output layers, b1: Bias of the hidden Neurons, b2: Bias of the output Neurons.

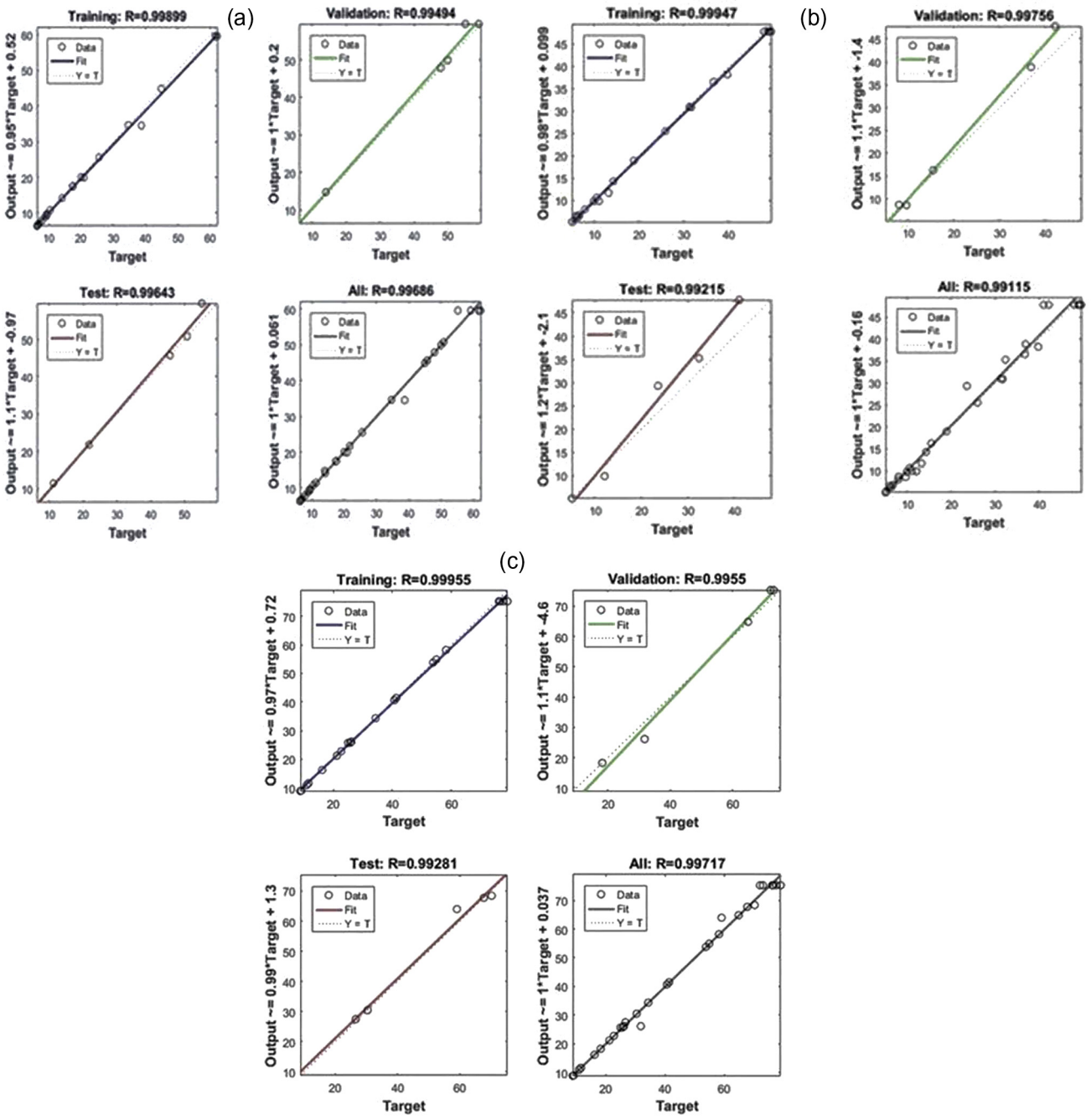

Fig. 5. Neural Network model with training, validation, test and all prediction set for (a) Amylase Activity of garbage enzyme, (b) Lipase Activity of garbage enzyme and (c) Protease Activity of garbage enzyme.

flexibility of the ANN based model in the prediction of the amylase, lipase and protease activity in garbage enzyme solution with minimum number of experimental runs.

3.7. Comparison between RSM and ANN models

In this study, the design of experiments was used to develop RSM and ANN model. The comparison on predictive capabilities of developed models were studied on the basis of various parameters namely correlation coefficients (R^2), root mean square error (RMSE), standard error of prediction (SEP), and absolute average deviation (AAD). The generalization capability of models can be verified by its prediction accuracy for a validation data set. The values of RMSE, SEP, and AAD for ANN were less than of those for RSM as observed from Table 5. These results indicate that the RSM model prediction has a greater deviation than the prediction by using the ANN model. This also means that the experimental data has been fitted with a high accuracy using the ANN model.

In addition, Fig. S3a, S3b, and S3c depict the experimental and predicted values for each experimental run to obtain the higher activity of amylase, lipase and protease in garbage enzyme solution respectively. From the Fig. S3a, S3b, and S3c, it is confirmed that the trained neural network has the values similar to experimental values. The ANN model predictions lie much closer to the line of

Table 5
Comparisons between predictive capabilities of RSM and ANN models.

Performance parameters	RSM			ANN		
	Amylase	Lipase	Protease	Amylase	Lipase	Protease
Correlation coefficient (R^2)	0.9825	0.9718	0.9792	0.9994	0.9989	0.9982
Root mean square Error (RMSE)	2.629	2.560	3.4118	0.4490	0.4604	1.004
Standard predicted deviation (SEP %)	8.800	11.018	7.97	1.5028	1.981	2.346
Absolute average deviation (AAD %)	7.536	7.13	6.997	1.4166	0.8095	2.780

perfect prediction compared to RSM model indicating considerably greater generalization of ANN model than the RSM model. Similarly, Shanmugaprakash and sivakumar (2013) reported that this higher predictive accuracy of the ANN can be recognized to its universal ability to estimate the nonlinearity of the system, whereas the RSM is restricted to a second-order polynomial. Rajkovic et al. (2013) suggested that care must be taken to select the process parameter range used for the training process as ANN model predictions are restricted within that range.

4. Conclusion

In the present study fruit dregs (6 g pineapple: 4 g citrus peels-20 min sonicated), molasses and water were fermented to produce garbage enzyme solution. The continuous production of garbage enzyme and its scaling up process need a globe optimized condition. The numerical global optimized conditions for co-production of amylase, lipase and protease in garbage enzyme solution were found to be pH (6), temperature (37 °C), agitation (218 RPM) and fermentation duration (3 days). The obtained garbage enzyme solution with higher hydrolytic enzyme activity can be used in solubilize the WAS and which in turn used to produce a sustainable energy through the anaerobic digestion process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.12.029.

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