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Statistical optimization of agro-industrial diets for the rearing of *Cydia pomonella* using response surface methodology

J. R. Gnepe[1], R. D. Tyagi[1*], S. K. Brar[1], J. R. Valero[1] and R. Y. Surampalli[2]

[1]INRS Eau, Terre et Environnement (INRS-ETE), 490, de la Couronne, CP 7500,
Québec, Canada G1K 9A9; Phone: (418) 654 2617; Fax: (418) 654 2600
[2]US EPA, P.O. Box-17-2141, Kansas City, Kansas, KS 66117
e-mail: gnepej@yahoo.fr; satinder.brar@ete.inrs.ca; tyagi@ete.inrs.ca;
josevalero@videotron.ca

Abstract. In this study, apple pomace and brewery wastewater were used as nutritive agents and as alternative substitutes for the ingredients (soya flour, wheat germ and yeast extract) without affecting the production of the diet. The quantity of agro-industrial waste added during production was based on a regime where the different nutrients were maintained as a constant, such as proteins (3.71 ± 0.09 g), carbohydrates (4.2 ± 0.12 g) and lipids (2 ± 0.08 g) based on their concentration in the standard diet. Various diets produced using different concentrations of waste and ingredients were tested using the culture of *Cydia pomonella* in order to optimize the diet in terms of nutrition and optimal viscosity (to facilitate assimilation of nutrients). Optimization of the rearing parameters was carried out using response surface methodology. This demonstrated that the brewery wastewater (BWW-SF) diet provided the best results for insect-rearing (81% hatching, 76% larvae and 51% adults) which was closer to the control diet (90% hatching, 80% larvae and 65% adults) and was more significant than the other diets (40–70% hatching, 45–50% larvae and 9–30% adults). In addition, the viscosity was higher in diets where the solids content was higher. The increase in viscosity was in line with the solidification of agar, which evolved rapidly over time and in relation to the solids present in the diet.

Keywords: Diet, surface response methodology, wastewater, agro-industrial waste & *Cydia pomonella*

INTRODUCTION

Studies on principal nutritional needs for the growth and reproduction of insects have been carried out since 1940 on various representative insects (Nation, 2001; Genc, 2002). The diets normally consist of synthetic nutritive and semi-synthetic substances of vegetable or animal origin (Arijs & De-Clercq, 2004; Cohen, 2004; Genc, 2006). Among the nutritive substances, soya flour, wheat germ and yeast extract have been used as sources of proteins, carbohydrates and lipids in the diet for the rearing of the codling moth (Cohen, 2004; Hansen & Anderson, 2006; Toba & Howell, 1991). These substances can be replaced by other compounds to reduce the cost of the diet while maintaining all of the nutritive substances necessary for the growth of codling moths.

The favourable growth of *Cydia pomonella* (Lepidoptera: Tortricidae) depends on the nutritional balance of different semi-synthetic substances (soya flour, wheat germ and yeast extract) and synthetic substances (sucrose, agar, methyl hydroxyl benzoate, rock salt, vitamins, ascorbic acid and water) as found in the standard diet (Chapman, 1998; Nation, 2001). The replacement of these ingredients must maintain or improve their nutritive potential and texture. The food imbalance is often responsible for the small size of and stress in the insects (Genc, 2002) which is produced at three levels: variation of the total quantity of introduced food; variation of the diets with a different nutritive balance; and efficacy of nutritive substances. Thus, to produce an economical diet without affecting the quantity and texture of the diet, apple pomace wastewater and brewery wastewater could be used to replace the nutritive characteristics (proteins, carbohydrates, lipids and minerals). In fact, the rich nutritive potential of wastewater and agro-industrial sludge has been exploited for the production of various value-added products (Tyagi et al., 2002; Cohen, 2003; Brar et al., 2009; Vu et al., 2004; Tarek et al., 2010; Gnepe et al., 2011). This substrate can be used as an ideal substitute for the replaceable ingredients (soya flour, wheat germ and yeast extract). Nevertheless, these new nutritive agents must be used in a methodical and concise manner. Hence, response surface methodology must be adopted to reduce the number of planned experiments and optimize the results (Tarek et al., 2010; Pham et al., 2009) which will make it possible to produce improved diets (in nutrition and texture) so as to allow improved growth of *Cydia pomonella*. In order to improve the texture of the produced diet, a study of agar, which is made up of complex polysaccharides (Lahay, 2001; Lahrech et al., 2005), and a responsible gelling agent for the solidification and viscosity of the diet remain significant elements for the availability of nutrients and assimilation of the diet.

The objectives investigated as a part of this study were:

- production of a diet for the rearing of the codling moth by using apple pomace and microbrewery wastewater;
- optimization of the culture of *Cydia pomonella* larvae using response surface methodology to determine the best diet in terms of nutrients (proteins, carbohydrates and lipid) and viscosity; and
- study of the properties of agar mixed with the diet as a function of time and temperature.

MATERIALS AND METHODS

Codling moth larvae

The eggs (20–25) of the codling moth, *Cydia pomonella (L)*, reared in the INRS-ETE laboratory (University of Quebec) were provided by BioTepp Inc. (Cap-Chat, Québec, Canada). The larvae were reared on the alternative diet, in a sterile environmental chamber (25 ± 1°C, 50 ± 0.5% moisture and L:D 16:8 h of photoperiod).

Culture media for rearing codling moth larvae

Diets used to rear the codling moth larvae included: (1) a control diet (a standard diet of BioTepp Inc) composed of semi-synthetic ingredients [6.85 g soya flour (SF), 1.6 g yeast extract (YE) and 2.2 g wheat germ (WG)], synthetic substances (0.55 g Vanderzan vitamin, 0.99 g Wesson salt mixture, 1 g sucrose, 0.5 g ascorbic acid, 1.3 g methyl-p-hydroxy benzoate) and 50 ml sterile milli-Q water; (2) an alternative diet composed of brewery industry wastewater (BWW) from Barberie (Quebec); and (3) apple pomace pulp waste (POM) from Lassonde Inc., Rougemont (Quebec) lyophilized as a substitute for the semi-synthetic ingredients.

The rearing of codling moth larvae was carried out on three alternative diets produced using different waste quantities: the initial quantity (noted 1) necessary for the replacement of ingredients on the basis of nutrients (proteins, carbohydrates and lipids), replacement as half (noted ½) and replacement as one third (noted 1/3) are set out in Table 1. The mass of waste necessary to replace the ingredients in the control diet was determined by Equation (1):

$$\text{Mass of waste} = \frac{\text{Ingredient mass x Nutrient concentration in ingrdient}}{\text{Nutrient concentration in waste}} \tag{1}$$

Table 1. Screening of waste and its major metabolic contents as alternatives substitutes for *C. pomonela* diets.

Sample	Weight of residual waste (g)		SF (g)	WG (g)	YE (g)	Carbohydrates (g)	Proteins (g)	Lipids (g)
Standard Diet	-	-	7±0.1	2.4±0.1	2±0.1	4.2 ±0.12	3.71±0.1	2±0.08
BWW (g)	1*	20±0.3	-	-	-	6.4±0.1	5.4±0.1	1.9±0.07
	1/2*	10±0.2	-	-	-	3.2±0.09	2.7±0.1	0.95±0.06
	1/3*	5±0.1	-	-	-	1.6±0.05	1.4±0.1	0.48±0.04
POM (g)	1*	26±0.3	-	-	-	6.5±0.11	4.2±0.2	1.82±0.05
	1/2*	13±0.3	-	-	-	3.25±0.08	2.1±0.1	0.91±0.02
	1/3*	7.5±0.2	-	-	-	1.88±0.07	1.2±0.1	0.53±0.01

*Quantity of waste added to diet (1 = initial; ½ = half and 1/3 = third)
±: Standard Error; n = 5
SF: Soya flour
WG: Wheat germ
YE: Yeast extract

During this study, several rearing parameters (Equations 2, 3 & 4) were tested (Table 3) for different diets comprising the brewery waste (BWW: experiment 9; BWW+SF: experiment 13; BWW+YE: experiment 10; BWW+WG: experiment 11; BWW+SF+YE: experiment 14; BWW+SF+WG: experiment 15; BWW+YE+WG: experiment 12; BWW+SF+YE+WG: experiment 16), apple pomace (POM: experiment 17; POM+SF: experiment 21; POM+YE: experiment 18; POM+WG: experiment 19;

POM+YE+SF: experiment 22; POM+WG+SF: experiment 23; POM+YE+WG: experiment 20; POM+SF+YE+WG: experiment 24) and apple pomace and brewery waste (BWW+POM: experiment 25; BWW+POM+SF: experiment 29; BWW+POM+YE: experiment 26; BWW+POM+WG: experiment 27; BWW+POM+YE+SF: experiment 30; BWW+POM+WG+SF: experiment 31; BWW+POM+YE+WG: experiment 28) plus the standard diet of BioTepp (experiment 8) and an experimental control diet (numbers 43–48).

$$\%\text{Hatching} = \frac{\text{Hatched eggs}}{\text{Total eggs}} \text{ x } 100 \qquad (2)$$

$$\%\text{Larvae growth} = \frac{\text{Adult larvae}}{\text{Total eggs}} \text{ x } 100 \qquad (3)$$

$$\%\text{Adult moth growth} = \frac{\text{Adult moth}}{\text{Total eggs}} \text{ x } 100 \qquad (4)$$

The insects reared on these diets exhibited a longer development time (1 month to 45 days) and limited longevity (6 to 10 days) as adults.

Viscosity

Waste and standard diet viscosity (all ingredients were mixed in 40 ml of milli-Q water without agar) were studied using a Brookefield DVII PRO rotational viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MY, the USA) equipped with Rheocalc 32 software. A SC-34 spindle (small sample adaptor) was used with a sample cup volume of 18/50 mL.

Viscosity studies enabled the consistency of the diet produced using waste to be determined and the quantity of nutrients required by larvae during rearing experiments to be estimated.

The viscosity profile of each diet was analyzed according to time and temperature. During production, 10 g of agar was mixed with 1000 ml of milli-Q water and sterilized in an autoclave at 121 ± 1°C/15 min.

Experimental plan for optimization of diets used for rearing the codling moth

The optimization experiments of diets produced using waste from POM and EMB for the rearing of the codling moth larvae were carried out using the following five parameters: concentration of apple pomace wastewater (POM); brewery wastewater (BWW); soya flour (SF); wheat germ (WG); and yeast extract (YE). Each variable was studied at four levels in different combinations. Response surface methodology was employed for the selection of experiments to determine the optimum values (Table 2).
Once the provisional optimal values were determined, a central composite design (CCD) was used to check the significance of the impact of each factor on the response. A central composite design of type 2^5 was employed at five levels, leading to 48 experiments, including 32 end points, 6 central points and 10 stars (α = 2). This distance from the centre of the design space (i.e. scaled value for α) of a circumscribed CCD are sometime negative in order to maintain the scaled value for α between the different levels. However, in the results given below, the negative numbers are

considered to be zero. The level of each factor and their codes and values are presented in Table 3. Each column in Table 3 represents the diets (number 1 at 48°C) for the nutrient constituents of pomace waste (POM), brewery waste (BWW), soya flour (FS), yeast extract (YE) and wheat germ (WG), along with the parameter of rearing, represented as egg hatching, larvae and adults given as percentages.

Table 2. Codes and values of independent variables of different levels of 2^5 factorial portions for central composite design (CCD) using response surface methodology (RSM).

Factor	Symbol	Levels				
		-2	-1	0	+1	+2
Pomace wastewater	POM	-6.5	0	6.5	13	19.5
Brewery wastewater	BWW	-5	0	5	10	15
Soya flour	SF	-3.5	0	3.5	7	10.5
Wheat germ	WG	-1.2	0	1.2	2.4	3.6
Yeast extract	YE	-1	0	1	2	3

After the realization of the experiments of central composite design, a regression equation of a second-order polynomial was adapted to the data (Equation 5).

$$Y = \beta_0 + \sum_{i=1} \beta_i x_i + \sum_{i=1} \beta_{ii} x_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} x_i x_j \tag{5}$$

Y = predicted response of dependent variable; X_i and X_j = independent variables influencing the response of Y; β_{ii}, *and* β_{ij} = dependent variables for the predicted response, β_0 = constant of quadratic equation; β_i = linear regression of each independent variable; β_{ii} = quadratic regression coefficient of regression for each independent variable; β_{ij} = coefficient of regression for interaction between two independent variables.

Statistical analysis

Analysis of the data, regression coefficient and surface response methodology was carried out via analysis of variance (ANOVA) using STATISTICA software (version 8; Statsoft.com). The degree of confidence in hypothesis testing for this study was fixed at 0.05.

Physico-chemical parameters

The concentrations of proteins in the samples were determined by the multiplication of the organic nitrogen present by 6.25. The organic nitrogen was obtained by calculating the [NH_4^+] and nitrogen present in the waste, and the diets were studied according to the standard methods (APHA, 2005). The measurement of total nitrogen in agro-industrial wastewater was performed using a nitrogen analyzer (Leco CINS-932, US) according to the standard methods (MENV, 2004; APHA, 2005).

Table 3. Study of rearing parameters for diets produced using RSM.

Diet	POM (g)	BWW (g)	SF (g)	WG (g)	YE (g)	% hatching	% larvae	% adults
1	0	0	0	0	0	13	8	0
2	0	0	0	0	2	18	10	0
3	0	0	0	2.4	0	23	15	1
4	0	0	0	2.4	2	33	20	5
5	0	0	7	0	0	28	25	10
6	0	0	7	0	2	36	28	13
7	0	0	7	2.4	0	45	34	15
8	0	0	7	2.4	2	90	80	62
9	0	10	0	0	0	66	56	14
10	0	10	0	0	2	68	60	16
11	0	10	0	2.4	0	71	63	20
12	0	10	0	2.4	2	76	65	27
13	0	10	7	0	0	79	76	32
14	0	10	7	0	2	83	69	37
15	0	10	7	2.4	0	88	65	45
16	0	10	7	2.4	2	75	55	28
17	13	0	0	0	0	52	43	19
18	13	0	0	0	2	55	46	25
19	13	0	0	2.4	0	58	53	28
20	13	0	0	2.4	2	60	58	32
21	13	0	7	0	0	68	66	25
22	13	0	7	0	2	60	58	19
23	13	0	7	2.4	0	70	55	21
24	13	0	7	2.4	2	73	49	26
25	13	10	0	0	0	51	48	22
26	13	10	0	0	2	55	35	19
27	13	10	0	2.4	0	53	37	20
28	13	10	0	2.4	2	52	33	21
29	13	10	7	0	0	58	40	24
30	13	10	7	0	2	59	35	26
31	13	10	7	2.4	0	62	31	25
32	13	10	7	2.4	2	64	29	23
33	-8.96	5	3.5	1.2	1	38	34	20
34	21.96	5	3.5	1.2	1	41	29	8
35	6.5	-6.89	3.5	1.2	1	40	35	22
36	6.5	16.89	3.5	1.2	1	39	30	9
37	6.5	5	-4.82	1.2	1	47	42	16
38	6.5	5	11.82	1.2	1	41	36	12
39	6.5	5	3.5	-1.65	1	50	45	18
40	6.5	5	3.5	4.05	1	49	44	13
41	6.5	5	3.5	1.2	-1.38	52	47	17
42	6.5	5	3.5	1.2	3.38	51	45	15
43C	6.5	5	3.5	1.2	1	58	52	22
44C	6.5	5	3.5	1.2	1	57	51	21
45C	6.5	5	3.5	1.2	1	58	52	20
46C	6.5	5	3.5	1.2	1	59	53	22
47C	6.5	5	3.5	1.2	1	58	53	22
48C	0	0	0	0	0	54.0	53.34	0.66

*The shaded sections correspond to the diets which yielded the best larval breeding *($P<0.05$).*

Soluble carbohydrates of the waste and the diets – essential elements for energy, mobility and the formation of the cuticle of the insects (Cohen, 2004) – were analyzed using the anthrone method (Moris, 1948; Bachelier & Gavinelli, 1966).

The lipids were determined in 2 g of sample, according to the gravimetric method followed by extraction by microwave (MARS RX, CEM Corporation, Matthews, NC, USA). This technique, more precise than the Soxhlet method (Salghi, 2008), makes it possible to analyze using a low volume (20 to 50 ml) of solvent mixture of ethyl cyclohexane/acetic acid in less time (50 min to 1h) at 80°C (Eskilsson & Björklund, 2000; Batista et al., 2001).

RESULTS AND DISCUSSION

Characterization of apple pomace and microbrewery used for the production of diets for the rearing of *Cydia pomonella*

The analysis of brewery wastewater (BWW) is presented in Table 1. BWW was rich in proteins (27% w/v), carbohydrates (32% w/v) and lipids (9.5% w/v) compared to pomace (16.15% w/v proteins, 27% w/v carbohydrates and 7% w/v lipids). The nutrient composition of BWW was related to the higher solids concentration (66.5 ± 8 g/L) and higher viscosity (770 mPa.s) compared to pomace (45 ± 5% w/v TS and 460 mPa.s viscosity).

For the production of diets tested for the rearing of the codling moth, the waste was used to replace the ingredients of the standard diet (soya flour, wheat germ and yeast extract) as a function of nutrient concentration (proteins, lipids and carbohydrates) as presented in Table 2. Based on these results, 13 ± 0.3 g of POM and 10 ± 0.2 g of BWW represent the nutrient concentrations closest to the standard diet (4.2 ± 0.12 g carbohydrates, 3.7 ± 0.7 g proteins and 2 ± 0.08 g lipid; Table 1). According to Toba & Howell (2006), the quantity of ingredients must be sufficient to provide the maximum quantity of nutrients necessary for larval growth.

Experimental plan for response surface methodology (RSM)

CCD of 2^5 with five factors (POM, BWW, SF, WG and YE concentration) and three parameters of rearing (hatching rate, larval growth rate and adults) as responses permitted to evaluate the selected experiments. The results of 42 combinations and 6 replicates of diets are shown in Table 3. Column 1 of Table 3 represents the diets produced using various components at different concentrations. Experiment 8 represents the control diet using only conventional ingredients in standard quantities (7 g SF, 2.4 g WG and 2 g YE). The concentrations with negative values obtained after selection of the model from Table 3 were regarded as null during the production of the diet.

Various diets produced using the waste as depicted in Table 3 showed that those corresponding to experiments 13 and 21 led to increased hatching of eggs (79% w/w and 68% w/w), larvae (76% w/w and 66% w/w) and adults (32% w/w and 25% w/w) compared to the standard diet (90% w/w hatching, 80% w/w larvae and 62% w/w adults). The effect of the addition of soya flour to BWW and POM was prominent compared to the contribution of wheat germ and yeast extract, taking into account the consistency of mass and concentration of nutrients brought about by these ingredients in the diets (Table 3). In order to perform the optimization of diets, larval growth was studied for surface response methodology, as this is an important parameter which allows consumption of diets during their growth.

Optimization of diets by response surface methodology (RSM)

The results of the RSM experiments are presented in Table 4, which shows the actual and predicted values for development of larvae on the produced diets. The residual values are the difference between the actual and predicted values. The growth of larvae increased by 9.6% and 8.7% for the BWW-SF and POM-SF diets compared to the control diet, which was 27.4%. Under these conditions, the growth of larvae on the diets comprising BWW-FS and POM-SF made it possible to increase the percentage of larval growth to 10%. These results were due to the rich nutrient content and viscosity of the diet, which was lower than the standard diet (Table 5). This depends on the viscosity and quantity of nutrients provided by SF and POM and BWW waste in the diets.

The statistical significance of the polynomial model of the second order was verified by ANOVA. This translated to the fact that the polynomial model of the second order comprising a coefficient of determination $R^2 = 0.80$ ensured an adjustment of the quadratic model of the experimental data.

Table 6 presents the effects of estimates of each variable and their interactions on the parameters of larval rearing with the regression coefficient of each component determined by regression analysis. The quadratic effect of the variables of POM (Q) and BWW (Q) were highly significant ($P<0.05$), followed by the quadratic effect of the concentrations of SF, WB and YE which were not statistically significant ($P>0.05$). The linear effects of the variables BWW (L) and FS (L) were significant ($P<0.05$) and the interaction between their effects were slightly significant ($P<0.05$). On the other hand, interaction between the linear effect of POM and BWW was considered highly significant because analysis of effect estimates showed a corresponding P value of <0.00001, which is lower than 0.05. The interactions existing between other factors were not significant ($P>0.05$), as presented in Table 6. The positive signs of the effects of variables indicated that the influence of variables on the growth of larvae were larger in the higher range (0.44 and 3.56). When the signs of the effects of the variables tested were negative, the influence of these variables on the growth of the larvae was larger in the lower range.

According to the data presented in Table 6 and as per the interactions which took place between BWW & POM, POM & soya flour (SF), BWW & SF and BWW & wheat germ (WG), the polynomial model of the second order is presented in Equation 6.

$$Y = 0.04\text{xPOM} + 6.67\text{x BWW} + 4.55\text{xSF} - 0.41\text{xPOMxBWW} - 0{,}17\text{xPOMxSF} - 0.25\text{xBWWxSF} - 0.63\text{ x BWWxWG} \qquad (6)$$

Table 4. Observed, predicted and residual values of larval rearing resulting from CCD experiments.

Diet	Factor by code unit					Observed values (%)	Predicted values (%)	Residual values (%)
	POM	BWW	SF	WG	YE			
1	-1	-1	-1	-1	-1	8.0	4.67	3.33
2	1	-1	-1	-1	-1	10.0	11.14	-1.14
3	-1	1	-1	-1	-1	15.0	18.36	-3.36
4	1	1	-1	-1	-1	20.0	31.96	-11.96
5	-1	-1	1	-1	-1	25.0	29.08	-4.08
6	1	-1	1	-1	-1	28.0	36.43	-8.43
7	-1	1	1	-1	-1	34.0	38.15	-4.15
8	1	1	1	-1	-1	80.0	52.62	27.38
9	-1	-1	-1	1	-1	56.0	59.38	-3.38
10	1	-1	-1	1	-1	60.0	55.22	4.78
11	-1	1	-1	1	-1	63.0	57.94	5.06
12	1	1	-1	1	-1	65.0	60.91	4.09
13	-1	-1	1	1	-1	76.0	66.41	9.59
14	1	-1	1	1	-1	69.0	63.13	5.87
15	-1	1	1	1	-1	65.0	60.35	4.65
16	1	1	1	1	-1	55.0	64.20	-9.20
17	-1	-1	-1	-1	1	43.0	48.52	-5.52
18	1	-1	-1	-1	1	46.0	45.62	0.38
19	-1	1	-1	-1	1	53.0	50.84	2.16
20	1	1	-1	-1	1	58.0	55.06	2.94
21	-1	-1	1	-1	1	66.0	57.31	8.69
22	1	-1	1	-1	1	58.0	55.28	2.72
23	-1	1	1	-1	1	55.0	54.99	0.01
24	1	1	1	-1	1	49.0	60.09	-11.09
25	-1	-1	-1	1	1	48.0	49.60	-1.60
26	1	-1	-1	1	1	35.0	36.07	-1.07
27	-1	1	-1	1	1	37.0	36.79	0.21
28	1	1	-1	1	1	33.0	30.39	2.61
29	-1	-1	1	1	1	40.0	41.01	-1.01
30	1	-1	1	1	1	35.0	28.36	6.64
31	-1	1	1	1	1	31.0	23.58	7.42
32	1	1	1	1	1	29.0	18.05	10.95
33	-2	0	0	0	0	34.0	36.71	-2.71
34	2	0	0	0	0	29.0	33.98	-4.98
35	0	-2	0	0	0	35.0	28.82	6.19
36	0	2	0	0	0	30.0	43.87	-13.87
37	0	0	-2	0	0	42.0	35.66	6.34
38	0	0	2	0	0	36.0	50.02	-14.02
39	0	0	0	-2	0	45.0	46.33	-1.33
40	0	0	0	2	0	44.0	50.35	-6.35
41	0	0	0	0	-2	47.0	49.28	-2.28
42	0	0	0	0	2	45.0	50.41	-5.41
43C	0	0	0	0	0	52.0	53.34	-1.34
44C	0	0	0	0	0	51.0	53.34	-2.34
45C	0	0	0	0	0	52.0	53.34	-1.34
46C	0	0	0	0	0	53.0	53.34	-0.34
47C	0	0	0	0	0	53.0	53.34	-0.34
48C	0	0	0	0	0	54.0	53.34	0.66

*The shaded sections correspond to the diets which yielded the best larval breeding *($P<0.05$)*.

Table 5. Design showing sets of each run and corresponding nutrient and viscosity.

Diet	Viscosity (mPa.s)	Carbohydrates (g)	Proteins (g)	Lipids (g)
1	0.9	0	0	0
2	29	0.7	0.7	0.1
3	32	1.1	0.7	0.3
4	33	1.8	1.32	0.33
5	34	2.4	2.4	1.4
6	38	3.1	3.1	1.5
7	47	3.5	3.1	1.7
8	142	4.2	3.71	2
9	770.5	3.2	2.7	1
10	803	8.9	3.4	1.1
11	806	4.3	3.4	1.2
12	814	5	4.2	1.3
13	823	5.6	5.1	2.4
14	825	6.3	5.7	2.5
15	838	6.7	5.8	2.6
16	857	7.4	6.4	3
17	460	3.25	2.08	0.91
18	468	3.95	2.78	1.01
19	483	4.35	2.78	1.21
20	489	5.05	3.48	1.31
21	492	5.65	4.48	2.31
22	496	6.35	5.18	2.41
23	499	6.75	5.18	2.61
24	427	7.45	5.79	2.91
25	987	6.45	4.78	1.91
26	1011	7.2	5.4	1.9
27	1018	7.6	5.4	2.1
28	1023	8.2	6.1	2.2
29	1026	8.8	7.2	3.3
30	1030	9.5	7.8	3.4
31	1039	9.9	7.8	3.5
32	1045	10.7	8.5	3.9
33	398	3.7	3.2	1.4
34	1589	9.5	6.9	3
35	409	3.96	3.1	1.41
36	1279	9.4	7.7	2.1
37	599	4.4	0	1.2
38	858	9.3	7.94	3.79
39	612	5.01	4.1	1.8
40	645	9.05	6.73	2.98
41	618	5.2	4.1	1.8
42	627	8.12	6.15	2.84
43C	624	5.6	4.4	1.9
44C	624	5.6	4.4	1.9
45C	624	5.6	4.4	1.9
46C	624	5.6	4.4	1.9
47C	624	5.6	4.4	1.9
48C	624	5.6	4.4	1.9

*The shaded sections correspond to the diets which yielded the best larval breeding *($P<0.05$)*

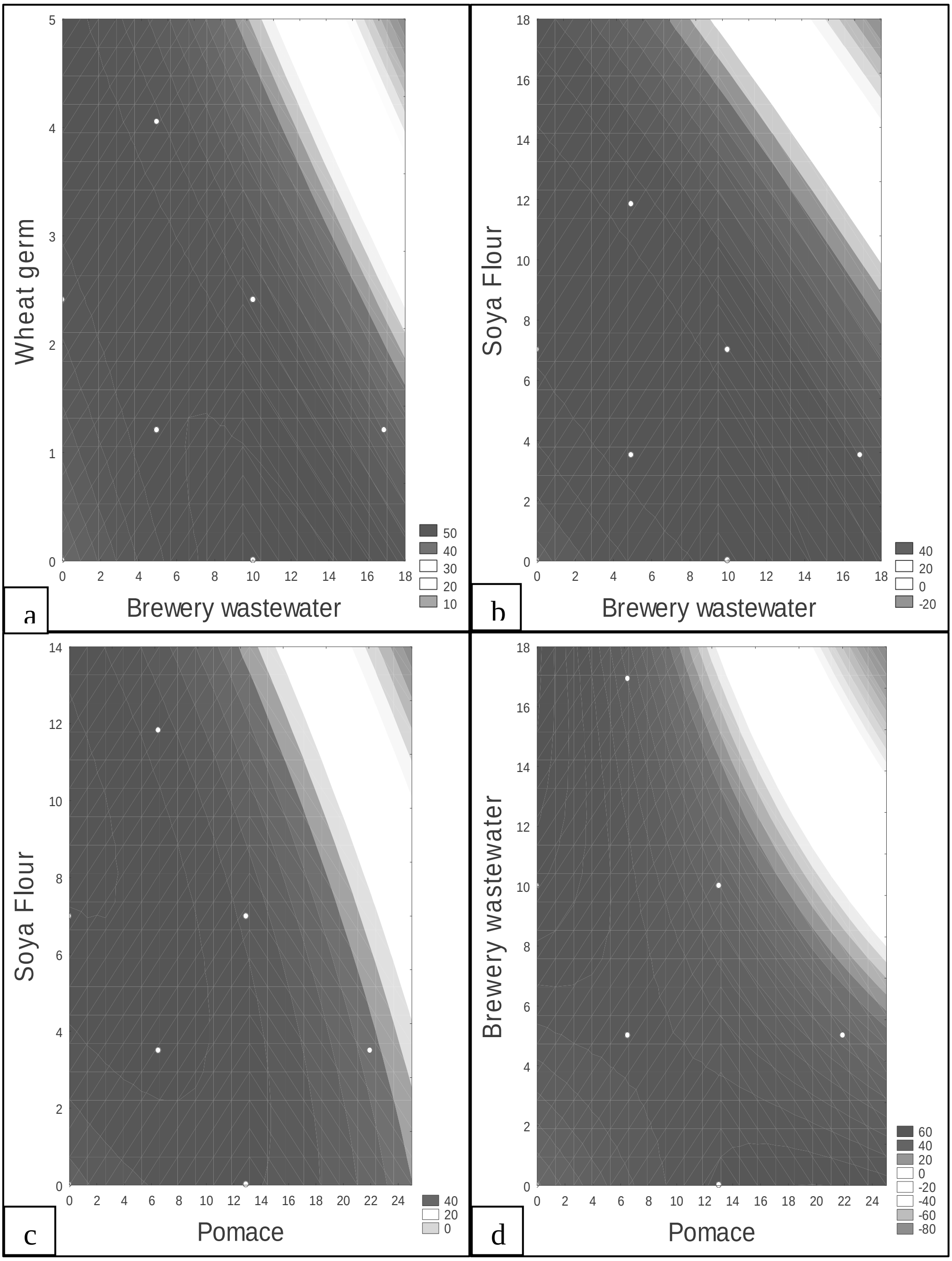
Table 6. Effect of estimation and regression coefficient of components of model fitted to data of CCD; $R^2 = 0.804$.

Component	Estimation effect		Regression coefficient	
	Effect	*P*	Coefficient	*P*
Constant	53.34	0.0	4.67	0.47
(1)POM (L)	-1.1494	0.691270	4.4	0.000001
POM (Q)	-6.3633	0.022616	-0.08	0.022616
(2)BWW (L)	6.3309	0.035686	6.67	0.000000
BWW (Q)	-6.0097	0.030467	-0.12	0.030467
(3)SF (L)	6.0364	0.044435	4.55	0.001425
SF (Q)	-3.7116	0.169806	-0.15	0.169806
(4)WG (L)	1.6910	0.559715	7.18	0.065162
WG (Q)	-1.7671	0.507586	-0.62	0.51
(5)YE (L)	0.4730	0.870028	4.47	0.33
YE (Q)	-1.2367	0.642134	-0.62	0.64
1L by 2L	-26.8125	0.000000	-0.41	0.00000
1L by 3L	-7.8125	0.026615	-0.17	0.027
1L by 4L	-5.6875	0.099236	-0.364	0.099236
1L by 5L	-4.6875	0.170793	-0.361	0.170793
2L by 3L	-8.6875	0.014662	-0.25	0.014662
2L by 4L	-7.5625	0.031391	-0.63	0.031391
2L by 5L	-5.3125	0.122406	-0.53	0.122406
3L by 4L	-2.3125	0.493493	-0.28	0.493493
3L by 5L	0.4375	0.896485	0.063	0.896485
4L by 5L	3.5625	0.294344	1.484	0.294

*The shaded sections correspond to independent variables that produced a highly significant effect during larval breeding *($P<0.05$).*

Analysis of optimization of experimental plan by RSM

Figure 1 shows the growth rate of the larvae on diets which consisted of various components (BWW-POM, BWW-SF, POM-SF and BWW-WG) presenting a significant interaction ($P<0.05$) as seen in Table 6. The results observed in these diets made it possible to demonstrate the effect of waste (POM and BWW) and ingredients (WG, SF and YE) on the growth of larvae. The results demonstrated that each variable corresponds to the intensity of the response. Fig. 1 showed that BWW-POM as a source of nutrients in addition to other ingredients



Fitted surface; Variable: % Larvae; 5 factors, 1 Block, 48 Runs; MS Pure Error = 1.1.

Figure 1. Response surface graph of larval breeding percentage for BWW-WG diet (a); BWW-SF diet (b); POM-SF diet (c); and BWW-POM diet.

(3.5 g of SF, 1.2 g of WG and 1g of YE) yielded better results with regard to the growth of larvae (48%). The results obtained with other diets were satisfactory (76% BWW+SF, 66% POM+FS and 65% BWW+WG). This was justified by the interaction between POM and BWW, whose effects were highly significant ($P<0.00001$), and the effects between BWW+SF, POM+SF and BWW+SF, which were also significant ($P<0.05$) for larval rearing (Table 6). These results were satisfactory in the sense that the apple industry and brewery wastewater possess higher viscosity. The diet mixture was in perfect synergy (related to the interaction between nutrients and particle size) with certain ingredients (SF and WG) which contributed to the optimal growth of the larvae.

Within the context of these results, BWW waste and SF presented a significant effect in interaction with other factors, taking into account higher nutrition content and TS concentration, which was more important than other factors. In fact, a good diet is a result of the synergy of nutrition and rheology in these components, which provided higher viscosity and adequate concentration of nutrients (proteins, lipids, carbohydrates and minerals) which was very significant for the growth of insects (Cohen, 2004; Hansen & Anderson, 2006). On the other hand, a larger quantity of the three nutrients led to poor growth of larvae due to higher viscosity (η>850 mPa.s and η<130 mPa.s) and excess nutrients which complicated food digestibility (Tables 3 & 5). Increasing viscosity led to decreased water availability and consequently lower water activity; in this case, it affected the enzymes (produced by the larvae) so that the larvae were not able to digest the nutrients present in the diet. This is in line with the results of Hansen and Anderson (2006), who reported that the quantity of nutrients can vary the viscosity and which can accordingly support the assimilation of the diet by the insects depending on viscosity. Likewise, diet ingredients in small quantities led to poor rearing of larvae due to the lower viscosity of the diets obtained, which was inappropriate for larval development. The diets, even in the presence of agar, solidified slowly due to the significant presence of water, which can lead to a proliferation of pathogens and dilution of the nutritive content of various diets. In fact, according to Cohen (2004), nutrient content is the most significant factor in a balanced insect diet.

The results observed in Table 3 show that the diets BWW+SF (experiment 13) and POM+SF (experiment 21) provided excellent results during rearing of the larvae – thus there were significant complementarities between the waste and soya flour (SF) for the growth of larvae. The optimized viscosity (1589 ± 34 mPa.s) was obtained in experiment 34 (POM+BWW+SF+WG+YE) and the optimized culture of larvae (62%) was in experiment 13 (BWW+SF), which was closer to that of the standard diet (90%). This diet showed a viscosity of 823 ± 29 mPa.s; the best optimized nutritive content (5.6 ± 0.19 g carbohydrates, 5.1 ± 0.17 g proteins and 2.4 ± 0.09 g lipids) was closer to that of the standard diet (4.2 ± 0.12 g carbohydrates, 3.7 ± 0.09 g proteins and 2 ± 0.08 g lipids). This is interesting in terms of the realization and economy of the process, as this waste is easy to handle and can replace two ingredients (WG and YE) in the standard diet without affecting larval growth. Likewise, the total substitution of three ingredients (SF, WG and YE) with two types of waste (BWW and POM) made it possible to obtain diets/experiments (9 and 17) that yielded a higher percentage of larvae (56% for BWW and 43% for POM).

Agar solution properties

Agar is an important ingredient, although not a nutrient per se, which determines the viscosity of the diet, and consequently the assimilation of the diet by the larvae due to higher viscosity. The solidification profile of the diet is shown in Fig. 2 as a function of viscosity. The diet viscosity increased with solidification and cooling time. Fig. 2 shows a rapid increase in the viscosity of diets (POM, BWW and control) as a function of time, in the presence of agar (Fig. 2a) and without agar (Fig. 2b). Fig. 2 shows that the viscosity of the diets produced with agar was higher (1500 mPa.s) compared to those without agar.

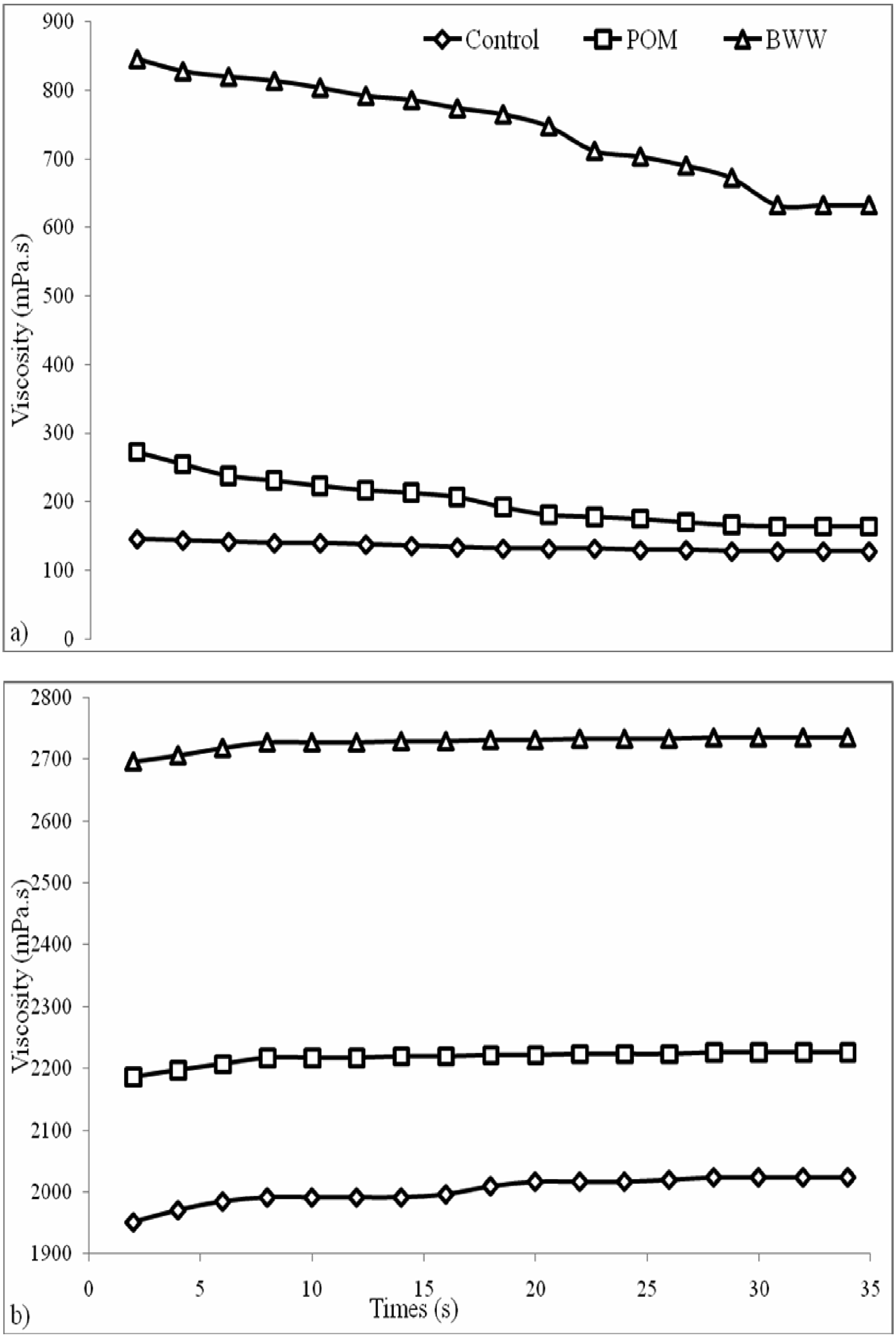
The diet with agar based on BWW produced higher viscosity (2696–2735 mPa.s) compared to the diet containing POM (2186–2226 mPa.s) and the control diet which provided lower viscosity (1952–2024 mPa.s; Fig. 2b). Fig. 2a shows that the viscosity of diets without agar decreased over time and that the viscosity of the diet based on BWW was considerably higher (an 846–632 mPa.s decrease) than that of the diet containing POM (a 273–164 mPa.s decrease) and the control diets (a 146–133 mPa.s decrease).

Viscosity increased slightly with the addition of agar, which solidified rapidly with the components present in the diet on the cooling of the diet. According to Lahrech et al. (2005), taking into account the polysaccharide composition, the viscosity of the agar followed Arrhenius’ law below a certain cooling temperature (Tc) and increased with a higher Tc (38°C) and then diverged as per the gelation temperature Tg (T=28°C). At a low temperature T_C, viscosity increased and diverged at the gelation temperature Tg (T=28°C).

Thus, viscosity differed from one diet to another due to the quantity and total solids concentration of the ingredients and waste, which influence the solidification of agar and increase the viscosity of diets. Fig. 2 shows that the viscosity of the BWW diet was higher (more than 500 mPa.s) than other diets due to the TS concentration of the BWW (64 g/L) which was higher than that of POM waste (46 g/L) and also compared to the ingredients of the control diet (6.89 g of SF, 2.4 g of WG and 1.7 g of YE). Moreover, BWW had a higher protein content compared to POM, which could have an additional effect on increasing viscosity taking into consideration hydrogen bonding between proteins and carbohydrates. The current interaction between these molecules allows the particles to be closer to one another, consequently making them more viscous in structure.

Thus, the higher TS concentration and protein content of BWW used to produce diets led to higher viscosity, and vice versa. These components can therefore reduce the quantity of water present in the diet and support weight increase on the solidification of agar with different diet components.

This is explained by the fact that the intrinsic viscosity $[\eta]$ of the diets at ambient temperature (28–40^{o}C) evolved in correlation with the molecular size of agar and the components present in the diet in relation to the equation of Mark-Houwink $[\eta] = 0.07\ M^{0.72}$, where M is the average molecular weight of the particles present in the diet (Rochas & Lahaye, 1989). This property holds for all fluid materials (i.e. Newtonian, Plastic, Dilatants), as their viscosity (η) depends on their molecular mass (M).



±: Standard Error; n = 5.

Figure 2. Viscosity profile of diets produced: (a) with agar; (b) without agar, as a function of time.

The viscosity behaviour of the diets was explained by the fact that in the absence of agar the profile decreased as a whole, as the diet components settled progressively according to their molecular weight and size. This does not support the homogenization of diets, which often presents several phases of settling, and the nutrients are far from the surface and inaccessible to the larvae. This is unfavourable for the assimilation of the diet by the larvae.

CONCLUSION

This study showed that a central composite plan and response surface methodology are effective methods for the determination of optimal conditions for the production of diets to improve the larval hatching rate of *Cydia pomonella*.

a) The central composite plan provided sufficient information for optimization to limit the number of individual experiments.

b) During the production of diets, waste (BWW and POM) replaced selected ingredients (soya flour, wheat germ and yeast extract) to maintain the quantity of nutrients (proteins, carbohydrates and lipids) present in the standard diet.

c) Multiple test experiments carried out using response surface methodology demonstrated that the BWW-SF diet yielded higher larval results (75% hatching, 62% larvae and 42% adults), closer to that of the standard diet (90% hatching, 80% larvae and 70% adults) due to the quantity of nutrients present (5.6 g carbohydrates, 5.1 g proteins and 2.4 g lipids) and viscosity (823 mPa.s) of the diet, which supported the assimilation of the diet by the larvae.

d) The viscosity was highly dependent on the solidification of agar and the mass composition of the diet. The diet solidified rapidly from 40–30°C, between 30 seconds and 1 minute depending on the concentration of these components.

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