Selection of Alcohols Through Plackett–Burman Design in Lipase-Catalyzed Synthesis of Anthranilic Acid Esters

C.V. Suresh Babu and S. Divakar*

Fermentation Technology and Bioengineering, Central Food Technological Research Institute, Mysore-570013, India

ABSTRACT: Lipase-catalyzed synthesis of esters of anthranilic acid was attempted by employing alcohols of carbon chainlength C_1–C_{18} using Plackett–Burman experimental design. Of the alcohols employed, methanol, decanol, cetyl alcohol, and stearyl alcohols showed 99.9% significance. Esterification of anthranilic acid with methanol gave the highest yield at 45.6%. This study allows the selection of better alcohols for esterification of anthranilic acid.

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KEY WORDS: Anthranilic acid esters, Plackett–Burman design, methyl anthranilate and porcine pancreas lipase.

Esters of aromatic carboxylic acids are difficult to synthesize by enzyme catalysis. Because of their bulky group, acyl transfer of aromatic carboxylic acids is difficult. Very few reports are available on the enzymatic preparation of esters of aromatic carboxylic acids. Esters of anthranilic acid are important flavor and fragrance compounds. Some of the known esters are methyl anthranilate, ethyl anthranilate, and butyl anthranilate, which possess sweet and orange odor with a pungent taste (1). Lipase-catalyzed synthesis of anthranilic acid esters of methanol, ethanol, and butanol was studied by Kittleson and Pantaleone (2). They used a biphasic solvent system for preparing these esters and employed lipase from *Candida cylindracea* in solvents like hexane, heptane, and ethyl acetate.

In order to determine the effectiveness of esterification of anthranilic acid using alcohols of carbon chainlength C_1 to C_{18}, Plackett–Burman (PB) design (3) was employed in the present work. PB design has been employed in several cases where selection of important variables from a set of a wide range of variables is required. This design has the advantage of limiting the number of experiments that are to be performed. Chan and Kavanagh (4) studied the effect of different components on liquid detergent formulations by employing PB design. Payot *et al.* (5) employed a fractional factorial experimental design to optimize batch and continuous fermentations for the production of lactic acid. Srinivas *et al*. (6) studied the production of α-galactosidase by screening several culture parameters using PB design in solid-state fermentation. However, this design has not been applied to lipase-mediated synthesis of esters so far. The present work has attempted to select the alcohol that gives the best ester yield with anthranilic acid in the presence of porcine pancreas lipase (PPL).

*To whom correspondence should be addressed.
E-mail: divakar@cscftri.ren.nic.in

MATERIALS AND METHODS

PPL, a Type-II crude preparation (Steapsin), was obtained from Sigma (St. Louis, MO). Esterification activity was determined by an esterification procedure (7). Reaction mixture (3 mL) consisting of butyric acid (0.16 M) and butanol (0.32 M) in heptane was incubated at 50°C with a specified amount of PPL for definite periods of incubation. Decrease in butyric acid content was measured by titrating the reaction mixture against standard NaOH. Esterification activity unit was defined as mol of butyric acid reacted per min per mg of enzyme. Specific activity for PPL was found to be 0.0478 mol/min·mg of enzyme.

Anthranilic acid was from Loba Chemie Ltd. (Bombay, India). The following straight-chain alcohols were purchased from SD Fine Chemicals (Bangalore, India): C_1–C_6, C_8, C_{12}, C_{16}, and C_{18}. Solvents were purchased from Qualigens Ltd. (Bombay, India). All liquid alcohols and solvents were distilled once before use.

The PB design chosen for the study is a 2-run design (n = 12) with 11 variables (K). It is a two-level fractional factorial design for studying $K = n - 1$ variables in n runs. The design projects three replicates of a full 2^2 design in any 2 of the 11 variables. The 11 variables studied were alcohols of carbon chainlength C_1–C_{18}. The highest (+1) and lowest (–1) levels chosen for the alcohols were based upon previous experiments carried out in our laboratory. This indicated optimal concentration for highest and lowest values that could be employed for a particular enzyme concentration. The lowest level for the study was a nonzero value of 0.001 mol and the highest was 0.005 mol, corresponding to the coded levels of –1 and +1, respectively.

Data analysis was carried out as described by Akhnazarova and Katarov (8). The first row in the design matrix was obtained from Akhnazarova and Katarov (8) for n = 12 and K = 11 and given as + – + – – – + + + – +. The remaining design matrix was generated row (or column)-wise from the first one by moving the elements of the row (or column) to the right (or down) one position and placing the last element of the first row (or column) in the first position. A third row (or column) was produced from the second similarly and the process continued until row (or column) K was generated. A row of minus signs was added to complete the design (4,9).

TABLE 1
Matrix of Plackett–Burman Design with Experimental and Predicted Yields

Number of carbon atoms of alcohol											Experimental yield (mmol)[a]	Predicted yield (mmol)
C_1	C_2	C_3	C_4	C_5	C_6	C_8	C_{10}	C_{12}	C_{16}	C_{18}		
1	–1	1	–1	–1	–1	1	1	1	–1	1	6.8	6.8
1	1	–1	1	–1	–1	–1	1	1	1	–1	4.4	4.7
–1	1	1	–1	1	–1	–1	–1	1	1	1	3.7	3.9
1	–1	1	1	–1	1	–1	–1	–1	1	1	3.8	3.8
1	1	–1	1	1	–1	1	–1	–1	–1	1	2.4	2.7
1	1	1	–1	1	1	–1	1	–1	–1	–1	3.1	3.4
–1	1	1	1	–1	1	1	–1	1	–1	–1	0.0	0.2
–1	–1	1	1	1	–1	1	1	–1	1	–1	3.9	3.3
–1	–1	–1	1	1	1	–1	1	1	–1	1	2.8	1.2
1	–1	–1	–1	1	1	1	–1	1	1	–1	2.7	3.0
–1	1	–1	–1	–1	1	1	1	–1	1	1	4.8	4.5
–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	1.1	1.4

[a]The results are an average from two experiments.

The experiments were then carried out in random order, and responses of values of each dependent variable for each run were determined and tabulated. The next two steps require calculating the effect of each independent variable in going from a low (–1) level to a high (+1) level and estimating the statistical significance of each of the independent variables with respect to the dependent variables. All the calculations were carried out from a computer program written specifically for this purpose using a Casio Personal Computer FX890 P. A copy of this program is available from the authors on request. The method of input of the experimental data and evaluation of coefficients and their significance are shown in the Results and Discussion section.

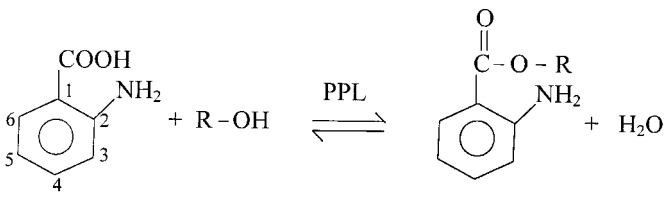
Based on the experimental design (Table 1), esterification reactions were carried out by taking anthranilic acid (0.035 mol) and alcohols C_1–C_{18} (0.001–0.005 mol) in a 50-mL chloroform and 100-mL hexane (1:2) mixture in a 250-mL round-bottomed flask with 250 mg PPL. Reactions were conducted in an experimental setup where continuous removal of water was maintained (10) and stirred for an incubation period of 72 h. The reaction mixture was refluxed at the boiling temperature (60°C) of the solvent system employed, and the solvent vapors were allowed to pass through a bed of desiccant after condensation, before being drained back into the reaction mixture. This cycle was repeated at the rate of 10 times per hour for a period of 72 h. The solvent system chosen was such that it formed an azeotrope with the water of reaction generated, and this procedure rendered the solvent system dry throughout the reaction system. Accumulation of water of reaction in the reaction mixture resulted in transesterification involving water that reduced the yield. Esterification yields were calculated by titrating the aliquots of reaction mixture against standard NaOH (11–13), which gave the decrease in acid content. Apart from the design experiments, a few experiments were performed for validation, based on the high statistical significance for some alcohols obtained. In these experiments, 0.02 mol of anthranilic acid and 0.02 to 0.2 mol alcohols were employed. Some of these experiments were conducted with 0.1 mL, 0.1 M pH = 7.0 phosphate buffer.

Products were monitored by thin-layer chromatography (TLC) and by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The solvent system employed for TLC was heptane/ethyl acetate (70:30). ^{1}H NMR spectra were recorded on a Brüker-AMX 400 NMR instrument (Bangalore, India) operating at 20°C. About 50–100 scans were accumulated for each sample with a total recycle time of 2.4 s. Deuterated chloroform ($CDCl_3$) was used as the solvent, and the chemical shifts were referenced to tetramethylsilane to within ±0.01 ppm.

RESULTS AND DISCUSSION

The objective of the present study was to develop a commercially viable method for preparation of anthranilic acid esters using lipases. Among the three lipases studied [*Rhizomucor miehei*: Lipozyme IM20 (Novo Nordisk, Bagsvaerd, Denmark) and Chirazyme (Boehringer Mannheim, Mannheim, Germany) and PPL], PPL was found to give better yields (data not shown). Also, PPL was stable at the reaction temperature (60°C) employed for incubation periods up to 15 d. In addition, PPL was the cheapest lipase among all the three lipases employed.

Reactions were carried out in a random order according to the levels of the alcohols indicated in the design. The following straight-chain alcohols were employed for the reaction (Scheme 1): C_1–C_6, C_8, C_{10}, C_{12}, C_{16}, and C_{18}. Each run contained six highest coded levels (+1) and five lowest coded levels (–1) of different alcohols (Table 1). Total amount of alcohols in the reaction mixture was 0.035 mol, and an equimolar



R = C_1–C_{18}

SCHEME 1

concentration of 0.035 mol of anthranilic acid was chosen for complete reaction to occur.

The experimental and predicted yields, along with the experimental design, are shown in Table 1. The experimental results of PB design were subjected to statistical analysis that yielded the coefficient and *t* values. The coefficient values (A_i) for each alcohol employed were determined using Equation 1. For $i = 0$, a dummy level of +1 was used, and the coefficient value obtained was designated as A_0.

$$A_i = 1/n \sum_{i=0}^{n} (x_i k_i) \quad [1]$$

where A_i = coefficient values; x_i = experimental yield; k_i = coded value of each alcohol corresponding to the experimental yield x_i; n = number of experiments.

Predicted yields (y_i) were determined using Equation 2:

$$y_i = \sum_{i=0}^{n} k_i A_i \quad [2]$$

Error mean square (Se^2) was determined from the sum of square of difference between experimental and predicated yields from which estimated error (S_b) was evaluated using Equation 3.

$$S_b = \sqrt{Se^2/n} \quad [3]$$

Student test *t*-values for each alcohol were determined from Equation 4.

$$t = A_i / S_b \quad [4]$$

Table 2 shows the coefficient values and the statistical data.

Methanol, decanol, cetyl alcohol, and stearyl alcohols showed 99.9% significance. Butanol and hexanol showed 99.5% significance, and propanol and pentanol showed 90% significance. The other alcohols showed significance less than 90%.

In order to validate the model, esterification experiments were carried out by considering individual alcohols according to the order of significance of their coefficients. Table 3 shows the results obtained. Generally it was observed that alcohols with 99.9% significance levels showed better yields, with highest yield from methanol followed by stearyl alcohol and decanol.

TABLE 2
Coefficients and Statistical Data[a]

Alcohol	S_e^2	Coefficients		*t* value	Significance (%)
	0.0829	A_0	3.41		
Methanol	0.0864	A	0.696	5.75	99.9
Ethanol	0.0864	A_2	–0.105	–0.867	80.0
Propanol	0.0846	A_3	0.223	1.843	90.0
Butanol	0.0864	A_4	–0.472	–3.9	99.5
Pentanol	0.0852	A_5	–0.217	–1.79	90.0
Hexanol	0.0852	A_6	–0.438	–3.62	99.5
Octanol	0.0858	A_8	0.057	0.471	80.0
Decanol	1.1685	A_{10}	0.863	7.132	99.9
Lauryl alcohol	0.0864	A_{12}	0.171	1.413	80.0–90.0
Cetyl alcohol	0.0858	A_{16}	0.525	4.338	99.9
Stearyl alcohol	0.0864	A_{18}	0.687	5.677	99.9

[a]S_e^2, Error mean square.

TABLE 3
Data on Yields of Anthranilic Acid Esters[a]

Alcohol[b]	PPL (mg)	Ester yield (%)	Significance (%) of the coefficient
Methanol	250	23.5	99.9
Decanol	400	11.7	99.9
Cetyl alcohol	250	5.5	99.9
Stearyl alcohol	500	13.7	99.9
Butanol	500	4.5	99.5
Pentanol	500	6.8	90.0
Lauryl alcohol	250	6.5	80.0–90.0
Octanol	250	3.6	80.0

[a]Anthranilic acid: 0.02 mol. Phosphate buffer: 0.1 mL, 0.1 M pH = 7.0.
[b]Alcohol: 0.02 mol.

The reaction between anthranilic acid and methanol was studied in detail, because, of all the alcohols, methanol showed higher conversion yields besides showing 99.9% significance. An optimal enzyme amount of 250 mg of PPL was required for the reaction. For methanol, higher amounts of PPL, greater than 250 mg, did not increase the yield. Experiments were conducted at different concentrations of methanol (0.01, 0.02, 0.05, 0.1, and 0.2 mol) corresponding to methanol/anthranilic acid ratios of 1 to 20. Figure 1 shows the reaction profile of ester yield at different methanol concentrations employed. At lower concentrations of methanol, esterification was low. This may be due to evaporation of methanol; however, at higher methanol concentrations (methanol/anthranilic acid ratio 5), conversion yields were good. A maximum yield of 45.6% was obtained at a methanol/anthranilic acid ratio 5. Further increase in methanol concentration to a methanol/anthranilic acid ratio of 10–20 decreased the ester yield.

Relative mobility factor (R_f) values determined by TLC were: anthranilic acid = 0.46, methyl anthranilate = 0.62, butyl anthranilate = 0.49, amyl anthranilate = 0.57, octyl an-

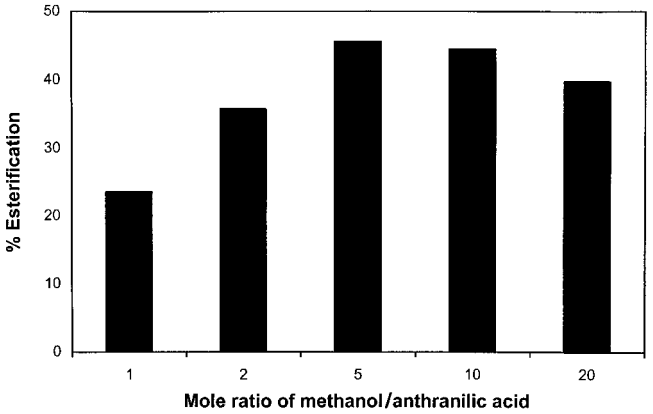


FIG. 1. Reaction profile of ester yield at different methanol concentrations. Reaction conditions: anthranilic acid—0.01 mol, porcine pancreas lipase—250 mg, phosphate buffer—0.1 mL, 0.1 M, pH = 7.0, and solvent—chloroform/hexane (1:2).

thranilate = 0.65, decyl anthranilate = 0.7, lauryl anthranilate = 0.7, cetyl anthranilate = 0.65, and stearyl anthranilate = 0.64. Chemical shift values from ^{1}H NMR spectra were: anthranilic acid: H-6 (7.92 ppm, 6.83 Hz), H-5 (7.31 ppm, 6.83, 6.01 Hz), H-4 (6.67 ppm, 6.01, 6.83 Hz), and H-3 (6.68 ppm, 6.01 Hz); methyl anthranilate: H-6 (7.25 ppm), H-5 (7.03 ppm, 7.25 Hz), H-4 (6.73 ppm, 8.16 Hz), H-3 (6.71 ppm, 8.16 Hz), and $-OCH_3$ 3.63 ppm. Both TLC and ^{1}H NMR indicated that anthranilic acid esters with different alcohols are formed in the reaction by lipase catalysis. ^{1}H NMR clearly indicated the formation of methyl anthranilate in the optimization reaction involving methanol.

Anthranilic acid exits as a zwitter ion in the native state with a positively charged $-NH_3^+$ and a negatively charged COO^-. Hence, it is difficult to esterify anthranilic acid . An earlier report (2) employed a biphasic solvent system for esterification. The present work describes gram level conversions of anthranilic acid using homogeneous reaction conditions and an experimental setup that aided continuous removal of water of reaction from the reaction mixture, leading to higher ester yields.

The PB design employed helps in arriving at the correct choice of alcohol from among a set of alcohols for obtaining maximal conversion. This information otherwise would require performance of a large number of experiments. This work has thus shown that the PB design employed for lipase-mediated esterification of acids like anthranilic acid can provide useful information regarding standardization of reaction conditions and choice of reactants for better yields.

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