

# Design of Experiments Improves Peptide Bond Yield from 20% to 76%



**L**iving matter is primarily made of proteins, so understanding how non-living matter can form proteins is critical to understanding how life emerged. The peptide bond, which is formed when the carboxyl group of one molecule reacts with the amino group of another molecule, releasing water, is the basic building block of proteins. In all of today's living cells, the ribosome, a large and complex molecular machine, serves as the site of biological protein synthesis. But, as life was emerging peptides had to be synthesized in a much simpler way. Researchers demonstrated decades ago that amino acids can be formed natu-

rally in conditions believed to have existed on earth when life was emerging and they have long tried to gain a better understanding of what conditions might be conducive to the formation of simple peptides. Peptide bond formation also has many important applications in pharmaceutical research and manufacturing since certain peptides have been shown to be effective in treating cancer, diabetes, infections and other diseases.

### Studying peptide bond formation

Researchers Professor Palwinder Singh, organic chemist, and Dr. Manpreet Bhatti, environmental engineer, at Guru Nanak Dev University, Am-

ritsar, India, recently worked to fine-tune the conditions that best promote peptide bond formation in an uncatalyzed aqueous phase reaction. In their first series of experiments, reaction of an equimolar solution of the amino acids (His) and proline (Pro) in solution in acetonitrile-water was studied as a function of temperature, pH, reaction time and concentration. The four factors of interest were varied one at a time while the yield was subjected to high-resolution mass spectroscopy to quantify the peptide bond formation by measuring the amount of the peptides Pro-His dipeptide, diketopiperazine 2, diketopiperazine 3, and tripeptide 4. However, it was difficult to optimize the conditions

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for the best yield of the dipeptide because the formation of diketopiperazine impedes the quantification of the formation of other peptides.

The researchers changed their approach to using aqueous solutions of Carbobenzoxy derivatized valine (N-Cbz-Val) and Glycylglycine methyl ester hydrochloride (Gly(OMe)-HCl) which prevents the formation of diketopiperazine. Gly(OMe) was used in place of glycine for easy isolation of the dipeptide methyl ester from the aqueous solution. However, glycine worked equally well for peptide bond formation. The one-factor-at-a-time (OFAT) approach was again used to investigate the effects of temperature, pH, reaction concentration and reaction time on yield. Over 75 runs, the reaction temperature ranged from 60°C to 120°C, the pH was varied from 3 to 10, the reaction time was between 30 and 300 minutes and the concentration ranged from 0.94 to 4.7M. Over 75 runs, the highest yield of peptides was 20%.

#### Interactions between factors

"We felt that we should be able to obtain a better yield than this," said Dr. Manpreet Bhatti, Assistant Professor in the Department of Botanical and Environmental Sciences at Guru Nanak Dev University. "I had a hunch that one or more interac-

tions between variables might be playing a role that was obscured by the OFAT method. A major problem with studying one factor at a time is that you cannot detect interactions between factors. By varying an individual factor you can find the optimal value of each one with all the others held constant. However, when you combine the supposedly-optimized values of each factor the results are often far less than optimal because of the ways that they interact with each other."

"Design of experiments provides a better approach that varies the values of all factors in parallel so it uncovers not just the main effects of each factor but also the interactions between the factors," Bhatti added. "This approach makes it possible to identify the optimal values for all factors in combination and also requires far fewer experimental runs than one factor at a time." Bhatti used Design-Expert® software from Stat-Ease, Inc. to develop a highly-fractionated two-level factorial screening experiment that evaluated 4 variables at a time, called a resolution IV design. The experiment required 10 runs to explore the design space, including investigating and evaluating the main effects of each factor as well as the two-factor interactions.



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#### Designing the experiment

Bhatti used a Design-Expert template to create an experimental design that requires the minimum runs needed for screening at this level of resolution. "We selected Design-Expert software because it is relatively easy to use compared to other statistical soft-

ware. Design-Expert walks the user through the process of creating a designed experiment. The user simply enters the factors and selects the type of design that fits his or her needs. The software provides feedback on the design, such as the number of runs required and the effects resolution. This makes it easy to quickly evaluate the pros and cons of different designs."

As shown in Figure 1, the factors in the experiment were reaction temperature (90° to 120°C), pH (5 to 7) reaction time (180 to 360 minutes) and concentration of reactants (2.5 M to 5.0M). A maximum yield of 74% of N-Cbz-Val-Gly(OMe) was obtained with a reaction temperature of 120°C, a pH of 5, a reaction time of 360 min and a reactant concentration of 2.5 M. Figure 2, shows the data analyzed by the Pareto chart which indicates a strong interaction of pH and temperature that had been masked in the one-factor-at-a-time experiment. The two-dimensional contour plot shown in Figure 3 further shows that the effect of temperature depends dramatically on the pH, which is the key to optimizing the reaction.

The results also showed a single peak in the chromatogram indicating a lack of racemization under the optimized reaction conditions. Racemization refers to the conversion of an enantiomerically pure compound (one where only one enantiomer is present) into a mixture of the enantiomers. A lack of racemization is valuable in pharmaceutical manufacturing applications because it eliminates the need for a subsequent operation to separate the enantiomers.

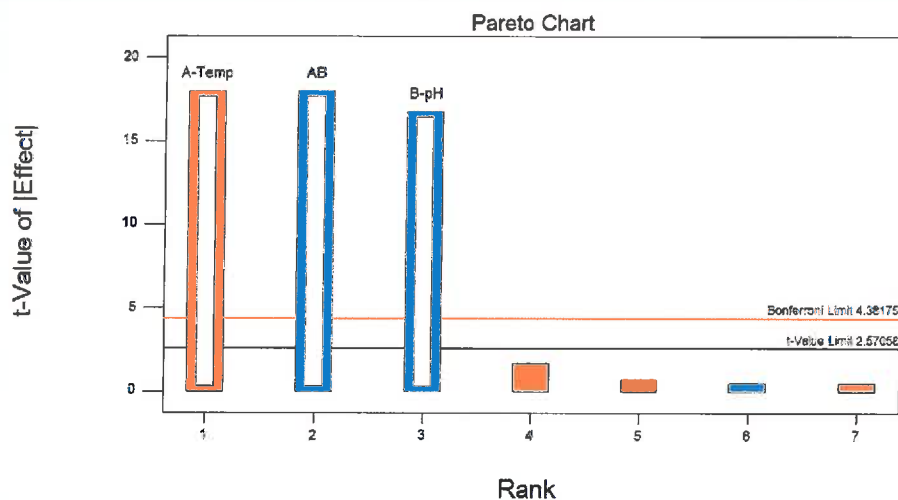
Figure 1

Select	Std	Run	Factor 1 A:Temp °C	Factor 2 B:pH	Factor 3 C:Time (min)	Factor 4 D:Conc	Response 1 Yield (%) Actual	Response 2 Yield (%) Predicted
	1	2	90	5	180	2.5	0.1	0.1
	2	3	120	5	180	5	70.0	72.0
	3	6	90	7	180	5	0.1	2.5
	4	4	120	7	180	2.5	0.1	2.5
	5	1	90	5	360	5	0.1	0.1
	6	8	120	5	360	2.5	74.0	72.0
	7	9	90	7	360	2.5	5.0	2.5
	8	10	120	7	360	5	5.0	2.5
	9	7	105	6	270	3.75	40.0	42.0
	10	5	105	6	270	3.75	44.0	42.0

Fractional factorial design showing four independent variables along with actual and predicted yield. The design is shown in standard order for illustration purposes, but was actually run in random order as shown in the Run column.

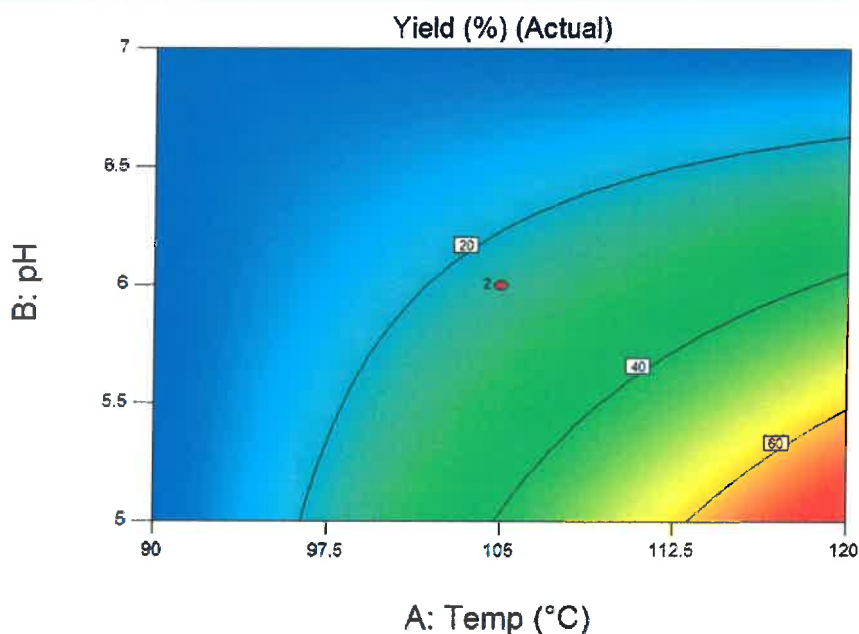
## FEATURE

Figure 2



Pareto chart showing the significant process variables.

Figure 2



Two-dimensional contour plot showing the effect of temperature and pH on compound yield.

a conventional method. These results were used to develop a synthetic protocol for production of peptides. The practicability of the method was also verified by synthesizing a pentapeptide. Starting with N-Cbz-Gly, the stepwise addition of Pro, Val, Ala and Ile resulted in the formation of a pentapeptide with a 55% overall yield.

In conclusion, DOE was used to demonstrate that temperature and pH function synergistically in the process of peptide bond formation. The optimized reaction was used to achieve sequence-specific and non-racemized synthesis of a tetrapeptide and pentapeptide at high yields. This is believed to be the first published report of constructing sequence-specific peptides in a noncatalyzed reaction. Besides demonstrating a possible pathway for the creation of proteins from nonliving matter, it may also prove to be an economical method for commercial peptide synthesis.



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### Building up the peptide chain

The optimized reaction conditions were used to produce a number of N-Cbz-dipeptides and also for a build-up of the peptide chain. The treatment of N-Cbz-Val-Gly(OMe) with NaOH in acetone-water, followed by the reaction of the resulting N-Cbz-

Val-Gly(OH) with L-leucine(OMe)-HCl resulted in the formation of N-Cbz-Val-Gly-Leu(OMe). Ester hydrolysis and the reaction of N-Cbz-Val-Gly-Leu(OH) with L-Ala(OMe)-HCl at 120°C and pH 5 resulted in N-Cbz-Val-Gly-Leu-Ala(OMe). Although the peptide was elongated in a stepwise

fashion, the sequence of amino acids in N-Cbz-Val-Gly-Leu-Ala(OMe) was verified with the help of the fragmentation pattern in the mass spectrum and nuclear magnetic resonance (NMR) spectra. For comparison of the optical rotation, N-Cbz-Val-Gly-Leu-Ala(OMe) was also prepared through

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