

285 amino acids. The size of the mutant library (N) can be calculated using the algorithm

$$N = 19^M \times 285! / [(285 - M)! \times M!]$$

as a function of M , the number of sites of amino acid substitution per enzyme molecule. For $M = 1$, the library would theoretically contain 5415 unique sequences. However, because of the degeneracy of the genetic code and the problems with the properties of certain sequences, the actual number would be significantly smaller. When $M = 2$, the theoretical number is approximately 15 million, and when $M = 3$, the theoretical number of *P. aeruginosa* variants exceeds 50 billion. This is a vivid example of the demands that these types of methods place on high-throughput screening systems for the sought-after characteristic, whether improved enantioselectivity, altered substrate specificity, or modified stability.

Here is the manner in which this formidable problem was confronted in searching for a *P. aeruginosa* lipase with improved enantioselectivity using the model reaction shown in Figure 11.9. This reaction was chosen because screening for one of the products, *p*-nitrophenol, can be performed very simply by absorbance spectroscopy. The wild-type enzyme exhibited very low enantioselectivity. The conditions for epPCR were chosen to introduce an average of one amino acid substitution per enzyme molecule per cycle of mutant generation; 2000 to 3000 mutants were screened and the process was repeated on the most enantioselective mutant from each cycle. Mutant A (S149G) showed an E value of 2.1 in favor of the *S*-isomer as compared to $E = 1.1$ for the wild-type enzyme. The results for the next three cycles were as follows: mutant B (S149G, S155L), $E = 4.4$; mutant C (V47G, S155L, S149G), $E = 9.4$; and mutant D (F259L, V47G, S155L, S149G), $E = 11.3$. Interestingly,

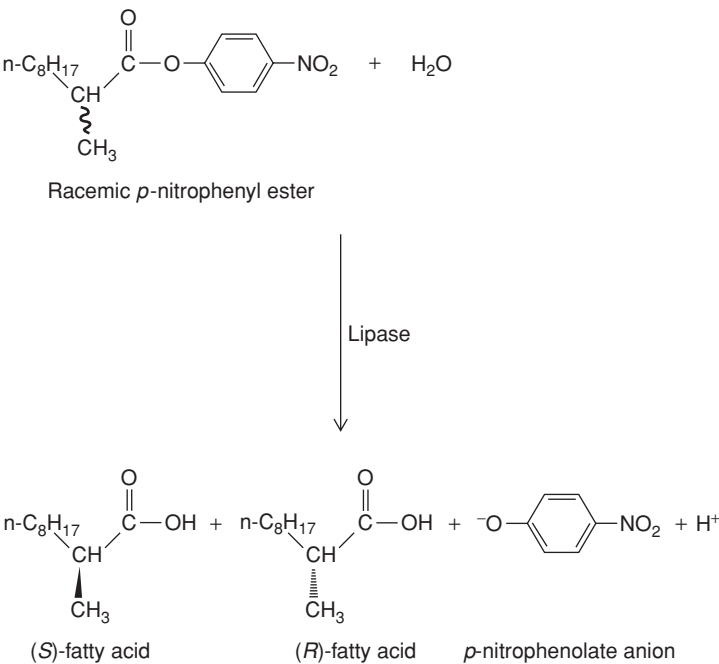


FIGURE 11.9

Assay for the enantioselectivity of a lipase by measuring the hydrolytic kinetic resolution of a racemic mixture of *p*-nitrophenyl esters. Enzyme-catalyzed hydrolysis of the racemic substrate yields two enantiomeric fatty acids and a *p*-nitrophenylate anion that absorbs strongly at 405 nm. To measure the enantioselectivity of each lipase variant, the assay was performed by comparing separately and pairwise the rate of *p*-nitrophenylate formation from the (*S*)- and (*R*)-substrates.