Systems biology

**Enzyme promiscuity prediction using hierarchy-informed multi-label classification**

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**Abstract**

**Motivation:** As experimental efforts are costly and time consuming, computational characterization of enzyme capabilities is an attractive alternative. We present and evaluate several machine-learning models to predict which of 983 distinct enzymes, as defined via the Enzyme Commission (EC) numbers, are likely to interact with a given query molecule. Our data consists of enzyme-substrate interactions from the BRENDA database. Some interactions are attributed to natural selection and involve the enzyme’s natural substrates. The majority of the interactions however involve non-natural substrates, thus reflecting promiscuous enzymatic activities.

**Results:** We frame this ‘enzyme promiscuity prediction’ problem as a multi-label classification task. We maximally utilize inhibitor and unlabeled data to train prediction models that can take advantage of known hierarchical relationships between enzyme classes. We report that a hierarchical multi-label neural network, EPP-HMCNF, is the best model for solving this problem, outperforming k-nearest neighbors similarity-based and other machine-learning models. We show that inhibitor information during training consistently improves predictive power, particularly for EPP-HMCNF. We also show that all promiscuity prediction models perform worse under a realistic data split when compared to a random data split, and when evaluating performance on non-natural substrates compared to natural substrates.

**Availability and implementation:** We provide Python code and data for EPP-HMCNF and other models in a repository termed EPP (Enzyme Promiscuity Prediction) at https://github.com/hassounlab/EPP.

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**Supplementary information:** Supplementary data are available at Bioinformatics online.

1 Introduction

Characterizing activities of enzymes plays a critical role in advancing biological and biomedical applications. While enzymes are traditionally assumed specific, acting on a particular molecule, many enzymes, if not all, have promiscuous activities acting on ‘non-natural’ substrates, ones other than those that the enzyme evolved to transform (D’Ari and Casadésus, 1998; Khersonsky et al., 2006; Khersonsky and Tawilik, 2010; Nobeli et al., 2009). Despite efforts in understanding types of promiscuity (substrate versus catalytic promiscuity) and cataloguing enzyme activities on various substrates in databases, comprehensive characterization of enzyme promiscuity remains elusive.

Several applications drive the development of computational tools to analyze enzyme promiscuity. The prediction of promiscuous products of Cytochromes P450 is enabled by techniques such as Metaprint2D-react (Adams, 2010), and PROXIMAL (Yousofshahi et al., 2015). Predicting products of metabolism, e.g. the MINEs database (Jeffryes et al., 2015) and BioTransformer (Djoumbou-Feunang et al., 2019), facilitates suggesting chemical identities for compounds collected through untargeted metabolomics. The approach proposed by PROXIMAL (Yousofshahi et al., 2015) was extended to enzymes other than Cytochromes P450 enzymes to facilitate annotation of untargeted metabolomics (Hassanpour et al., 2020) and for suggesting enzymatic activities responsible for catalogued products in metabolomics databases (Amin et al., 2019a, b). Prediction of putative enzymatic links, e.g. Selenzyme (Carbonell et al., 2018), XTMS (Carbonell et al., 2014) and ELP (Jiang et al., 2020), allows the construction of novel biosynthesis or biodegradation pathways. The study of enzyme promiscuity also elucidates the evolution of metabolic networks (Carbonell et al., 2011). Further, enzyme promiscuity can be tuned and exploited for novel protein engineering applications (Chen and Arnold, 2020).

We investigate in this paper data-driven approaches to predict enzyme classes, as defined via the Enzyme Commission (EC) numbers, that are likely to interact with a given query molecule. One approach is based on k-nearest neighbor (k-NN) similarity, where fingerprint similarity is calculated between the query molecule and
the 'natural' (native) substrates that are known to be catalyzed by the enzyme under natural selection and under cellular physiological conditions. The remaining four approaches are novel adaptations of several machine-learning models that frame the problem as multi-label classification, where each predicted label corresponds to an EC number. Each model has an architecture that embeds a different information-sharing paradigm across enzymes. Some models exploit hierarchical relationships encoded in the EC Nomenclature, which provides a tree-structured hierarchical classification of enzymes. 

We develop and evaluate machine-learning classifiers with a range of different patterns of information sharing across enzymes. 

We demonstrate the effective utilization of a large dataset of promiscuous interactions. We provide predictions for 983 enzyme classes, a huge increase over previous efforts that made predictions for four specific enzymes (Pertusi et al., 2017) using SVMs and active learning.

While substrate similarity is widely used for determining promiscuity in metabolic engineering practice (Pertusi et al., 2015), there is currently no large-scale systematic evaluation of the effectiveness of similarity in predicting enzyme promiscuity across enzymes or enzyme classes. Further, prior works have shown no consensus on a similarity level that deems a query molecule sufficiently similar to a native substrate. Our work offers such a study. Importantly, our work shows that machine learning outperforms similarity-based methods on several metrics including R-Precision (R-PREC), the metric that is most significant from a user’s perspective as it best correlates with downstream usefulness for an experimentalist.

Our experiments show that inhibitors are hard negative examples and that including inhibitor information during training consistently improves predictive power, particularly for EPP-HMCNF.

Our results show that all promiscuity prediction models perform worse under a realistic data split (Martin et al., 2017), when compared with a random data split, and when evaluating performance on non-natural substrates compared to natural substrates.

2 Materials and methods

2.1 Dataset

All positive and inhibitor molecules were collected from BRENDA, excluding co-factors because these metabolites are common among many enzymatic reactions. The Morgan fingerprint, with a radius of 2 and 2048 binary features (Rogers and Hahn, 2010), is used to represent each molecule. Compounds in BRENDA that could not be mapped to a specific molecular structure were discarded. By the end of the conversion process from names in BRENDA to Morgan fingerprints, we identified 25 872 positive pairings between molecules and EC numbers, based on 8295 unique molecules. Within this same set of molecules, we also identified 13 087 inhibiting interactions, based on 2165 unique inhibitors. Some enzymes had limited positive data. We focused on enzymes that had a minimum of 10 positive examples, so there would be enough data for both training and assessment. Using these inclusion criteria, our dataset consisted of 983 distinct EC numbers, of which 730 had at least one known inhibitor. All 983 EC numbers came from top-level classes 1-6, as we excluded the recently established top-level class 7 due to insufficient data. Of all positive pairings, only approximately 13% are associated with an EC class paired with a natural substrate. These pairings are associated with in vivo metabolism as specified under each EC Number’s ‘Natural Substrates Products’ section in the BRENDA database. The ratio of natural substrate to total interacting positive molecules, listed under the ‘Substrates/Products’ section, varied per EC Number (Fig. 1).

For every EC number, each of the 8295 molecules that were not known to be positive nor inhibitors are considered unlabeled in the BRENDA database. Per EC number, these unlabeled molecules were treated as negative examples during training, as were the inhibitors. Across enzymes, the ratio of positive-to-all molecules was on average 0.0032, with a standard deviation of 0.0044. The 10th percentile of this distribution was 0.0013, while the 90th was 0.0037.

Since inhibitors bind with enzymes, they might be considered ‘closer’ to the positive molecules and thus be more difficult (harder) to classify. In machine-learning applications, including such ‘hard negative’ examples can fine-tune the decision boundary between positives and negatives (e.g. Radenovic et al., 2016). We validated that inhibitor molecules across enzymes are, on average, more similar (based on Tanimoto score) to their respective positive molecules than a similar-size randomly selected set of unlabeled molecules (Supplementary File S1, Supplementary Section S1, Supplementary Fig. S1), thus confirming their status as hard negative examples.

Data was further organized in a tree hierarchy to match the structure of the EC nomenclature. There were 6 nodes at the class.
2.2 Models

We implemented one model based on fingerprint similarity and four machine-learning models. The machine-learning models (Supplementary Fig. S2) are presented in the order corresponding to the amount of information sharing they allow across enzymes: no sharing (predictions for each leaf node are developed independently); top-down hierarchical sharing (each leaf uses learned representations from parent); horizontal sharing (each leaf uses learned representations common across all enzymes); and horizontal-plus-hierarchical sharing (each leaf predictor uses shared representations, as well as representations common to leaves that share a parent).

2.2.1 k-NN similarity (no sharing)

Each EC Number-molecule pair in the test set was scored by computing the mean similarity between the test molecule and the k most similar positive molecules for the relevant EC Number in the training set. Similarity is evaluated via the Tanimoto score (Bajusz et al., 2015) on the Morgan fingerprints. Hyperparameter k was individually optimized for each EC Number via 3-fold cross-validation grid search on the training set, selecting the value with the best average precision score. The search considered the following values of k: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and ‘all’, where ‘all’ indicates that the mean similarity was computed between the test molecule and all positive molecules in the training set. A value of k = 1 corresponds to computing the maximum similarity, while the ‘all’ value corresponds to computing the mean similarity.

2.2.2 No-share RF (no sharing)

An independent Random Forest (RF) binary classifier (Breiman, 2001) was built for every leaf in the tree (distinct EC number). Each RF classifier was composed of 50 decision trees. At each internal decision node, a decision tree was trained to optimize the Gini metric while allowing for the selection of a random subset of features of size proportional to the square root of the number of features. During training, we selected model complexity hyperparameters (the minimum size of any tree’s terminal node) by performing a 5-fold cross-validation grid search. The grid search considered minimum terminal node sizes of 1, 3, 5, 10, 20, 50 and 100, selecting the value with best average precision score.

2.2.3 Hierarchical RF (greedy top-down hierarchical sharing)

This model (Fig. 2) uses a tree-like architecture that mimics the EC nomenclature hierarchy. A hierarchical cascade of random forests (RFs) was trained, with one RF predictor at each internal node and leaf node of the tree. Each of the six top-level enzyme categories had a root predictor trained to produce probabilistic predictions given data and binary labels. Then, an RF regressor at each lower-level node was trained to predict the residual error of the estimator at the parent node. The overall probabilistic prediction at a node is thus formed by adding its prediction to those from all preceding levels (thresholding to keep a valid probability in the unit interval). The hyperparameters of the RF predictors and RF regressors were selected in the same way as in No-Share RF.

2.2.4 Multi-label NN (horizontal sharing)

This model consists of a fully connected, Multi-Label Neural Network (NN) (Tsoumakas and Katakis, 2007). To make promiscuity predictions, each molecule’s input features are fed through a NN with four hidden layers to obtain a learned representation common to all enzymes, and then a final output layer with sigmoid activation produces enzyme-specific probabilistic predictions given this common representation. In this way, all enzymes share a common feature transformation trained to improve performance across all enzymes. However, unlike Hierarchical RF, there is no specific usage of hierarchical information that would prioritize sharing between enzymes of the same class, sub-class or sub-sub-class.

Each layer is fully connected with ReLU activation followed by batch normalization and dropout. Residual connections are present between the hidden layers. Binary cross entropy was used as loss. We used the Adam optimizer with a batch size of 12. The learning rate was manually set to 0.0002, and the model converged after 70 epochs. We cross-validated our model using random search (Bergstra and Bengio, 2012) across 12 pairs of dropout and layer size values that were sampled from uniform distributions between 0.01 and 0.6, and between 128 and 384, respectively. We selected values that maximized the average precision score.

2.2.5 EPP-HMCNF (horizontal plus hierarchical sharing)

We adopt HMCN-F, a state-of-the-art NN architecture for hierarchical multi-label classification (Wehrmann et al., 2018) for our task. HMCN-F consists of one global predictor, which predicts all labels.
in the hierarchy (producing a probability for each internal node and leaf node), and local predictors for each level in the hierarchy, which predicts labels only for that specific level. The global and local predictions are then merged into a final prediction. In our adaptation (Fig. 3), our model EPP-HMCNF was implemented with four global hidden layers and four local hidden layers, one dedicated to each enzyme class. Each layer is fully connected with ReLU activation followed by batch normalization and dropout. Probabilistic predictions are made via fully connected output layers with sigmoid activation. Residual connections are present between the hidden layers in the global predictor. EPP-HMCNF was trained similarly to the Multi-Label NN, for 70 epochs. The final prediction is obtained by equally weighting the predictions from the global and local predictors. Information is therefore shared across enzymes both in the horizontal fashion of the Multi-Label NN as well as in the vertical fashion across the EC hierarchy, as in Hierarchical RF. Unlike Hierarchical RF, however, training is not greedy across the hierarchy, but happens simultaneously at all levels.

2.3 Confidence weighting of unlabeled data

Providing a per-example weight (a scalar positive value) to make some examples more important during training is a common technique to overcome label balance issues or to account for unlabeled data that may unknowingly contain positive examples (Liu et al., 2003). We assign an overall weight \( w_{m,e} \) to each molecule-enzyme pair \((m,e)\) that is the product of a scalar similarity weight and a scalar label weight:

\[
 w_{m,e} = \text{SimW}_{m,e} \cdot \text{LabelW}_{m,e}
\]

The similarity weight \( \text{SimW}_{m,e} \) is used to denote our confidence in the provided positive or negative label. This value will be set to 1 for positive examples and inhibitors (since we are confident in their negative label). However, when a molecule is unlabeled for an enzyme in BRENDA, we assign it a negative label with a similarity weight between 0 and 1. This value is set to one minus the maximum similarity between the unlabeled molecule and all molecules associated with the corresponding enzyme in the positive set. Similarity is scored using the Tanimoto score for two molecular fingerprints. To overcome the challenge of negative labels far outnumbering positive examples, all examples are given a label weight \( \text{LabelW}_{m,e} \), which is the same for all examples of the same label. The label weight is set to 1.0 for positive molecule-enzyme interactions. For negative examples, it is set as:

\[
 \text{LabelW}_{m,e} = \frac{\text{NumPositive}_{m,e}}{\sum_m \text{SimW}_{m,e}}
\]

This weight enforces that the aggregate weight of samples in the negative class is equal to that of the positive class for each enzyme, thus balancing the influence of each binary class during training.

We provide these weights when training RF predictors using the ‘sample_weight’ keyword argument in Scikit-Learn (Pedregosa et al., 2011). For the NNs, we scaled the contribution of each molecule-enzyme to the loss function with its relative weight. If \( \hat{y}_{m,e} \) is the predicted probability and \( y \) the true binary label, then:

\[
 \text{loss} = \sum_{m,e} w_{m,e} \cdot \text{binaryCrossEntropy}(y_{m,e}, \hat{y}_{m,e})
\]

While we use weighted datasets for training to obtain more robust models, during model evaluation all examples are unweighted.

3 Results

We first trained and evaluated our models under the classic random train-test split. Using confidence weighting of unlabeled data, we trained the No-Share RF, Hierarchical RF, Multi-Label NN and HMCNF. We then evaluated the models, together with \( k \)-NN Similarity. We compared our methods against a baseline ‘Random’ model, where every enzyme-molecule pair in the test set is assigned a likelihood of interaction value that is selected from a uniform distribution between 0 and 1.

We perform our experiments on several test sets. The ‘Full Test Set’ comprises all enzyme-molecule-pair interactions that were saved for testing, corresponding to 20% of the total interactions collected from BRENDA. The ‘Inhibitor Test Set’ includes only the positive and inhibitor interactions, and none of the unlabeled ones, for each enzyme. The positive and inhibitor interactions were selected from the Full Test Set. Only 885 out of the 983 EC Numbers presented inhibitors, and only 671 EC Numbers presented inhibitor interactions among the data saved for testing. Therefore, only these 671 were considered for testing. The ‘Unlabeled Test Set’ contains positive and unlabeled examples, but excludes known inhibitors, in the same positive-to-total ratio as the Inhibitor Test Set. For all test sets, unlabeled molecules are uniformly sampled within each EC Number’s set. To generate a more competitive and realistic testing scenario, we split the molecular data into training and testing under a realistic split (Martin et al., 2017), and replicated some experiments under this split.

We measured the performance of our models via three metrics: Mean Area Under the Receiver Operating Characteristic (AUROC), Mean Average Precision (AP) and Mean R-Precision (R-PREC) (Manning, 2009). Each overall summary score is respectively computed by averaging the per-EC-Number scores across all EC Numbers in the corresponding dataset. These metrics were selected as they do not require thresholding and instead consider the ranking of positive examples relative to the unknowns in the test data. This is particularly well suited for our purposes, since the similarity models do not return results that can be interpreted as probabilities, and thus thresholding would have required ad hoc and likely unjustifiable assumptions. To compute R-PREC, we are given a list of candidate molecules as input with knowledge that some number \( R \) of them is truly relevant, and then we compute the precision among the \( R \) top-scoring candidates as ranked by the predictor. A model with high R-PREC has a high probability of ranking positive molecules ahead of unknown molecules. The top-ranked molecules are naturally the first ones that the user will consider for experimental testing. Therefore, a model with high R-PREC on a test set is most likely to be useful for end users.

3.1 Comparing the performance of models trained without known inhibitors

To train without including inhibitor information, inhibitors are treated as unlabeled molecules and are assigned similarity-based confidence weights. Figure 4 A–C shows a comparison of all models for each metric. Each score is reported with \( \pm 1 \) standard deviation across all EC Numbers. The results show that \( k \)-NN Similarity is a strong baseline for our classification task, thus confirming that substrate similarity is a valuable tool to predict promiscuity. However, similarity is outperformed by most machine-learning techniques. Increasing information-sharing across EC Numbers improves performance, with EPP-HMCNF showing the best mean scores across AP and R-PREC and tying with Multi-Label NN in terms of
AUROC. Hierarchical RF performs worse than No-Share RF with regards to all metrics. The greedy training of Hierarchical RF may limit its ability to fix any mistakes made at higher levels of the hierarchy. Notably, the ±1 standard deviation intervals are overlapping across all models, indicating that the difference in performance across models is smaller than the difference across ECs.

We also measure the relative performance of our models by ranking them on each of the 983 binary classification tasks (one per EC Number) and then summarize the results by computing the average rank. Whenever two or more models tie in rank on a particular EC Number, we assign to all disputing models the average of the disputed ranks; this occurs more frequently on R-PREC scores, since its computation considers only a handful of predictions per EC Number. We repeat this for each of the three performance metrics (Fig. 4D). EPP-HMCNF ranks best for AP and R-PREC, tying with Multi-Label NN with regards to AUROC, consolidating itself as best model for this task. Overall, the results in Figure 4D are consistent with those in Figure 4A–C: models with higher scores in Figure 4A–C generally have lower (i.e. better) rank in Figure 4D.

We analyzed the average performance of the k-NN and EPP-HMCN-F models for each EC Class (Supplementary File S1, Supplementary Section S4, Supplementary Fig. S3). We found that some enzymes are easier to characterize than others. Class 3 hydrolase enzymes and Class 5 isomerase enzymes are consistently easiest to classify. Furthermore, we found that EPP-HMCN-F outperforms k-NN similarity for all classes.

We analyze the average performance of the models across EC Numbers as a function of the number of positive examples. We evaluate the performance of EC Numbers with the most and least number of positive examples (Supplementary File S1, Supplementary Section S5). We found that some enzymes are easier to characterize than others. Class 3 hydrolase enzymes and Class 5 isomerase enzymes are consistently easiest to classify. Furthermore, we found that EPP-HMCN-F outperforms k-NN similarity for all classes.

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3.2 Training with inhibitor information
To evaluate the impact of including known negative examples in the form of inhibitors, we retrained our models including inhibitor information, setting the similarity weight to 1 for each inhibitor. We then perform two sets of experiments. First, we test the models on the ‘Full Test Set’ and compare our findings against the result from the no-inhibitor models. Table 1(A) summarizes the differences among the models and reports the percentage of EC Numbers for which the use of each training technique yields a better score. Hierarchical RF and HMCN-F are the models that consistently benefit the most from training with inhibitors, with EPP-HMCNF experiencing a significant boost for all metrics.

Next, we considered the same metrics on the Inhibitor Test Set. The goal of using this set is to show the capacity of each model in distinguishing between positive molecule and inhibitor molecules, the only true negative examples in our dataset. We also tested our models on the Unlabeled Test Set. Since this set contains positive and unlabeled interactions with the same ratio as the ratio between positive and inhibitor interactions of the Inhibitor Test Set, any difference in scores between the Inhibitor and the Unlabeled Test Sets can be attributed entirely to group differences between the inhibitor molecules and unlabeled molecules. Results on the two test sets for all models, trained with and without inhibitor information, are shown in Figure 5. In the Inhibitor Test Set and in the Unlabeled Test Set, the positive-to-total ratios across EC Numbers are much higher than in the Full Test Set. This is important to note since AP and R-PREC scores correlate with the positive-to-total ratio. For each of these sets, the average ratio is 0.579, with a standard deviation of 0.199, a 10th percentile of 0.286, and a 90th percentile of 0.833.
### Table 1. Evaluation of training without and with inhibitors

|                  | AUROC | AP | R-PREC |
|------------------|-------|----|--------|
|                  | No-Share RF | Hierarchical Share RF | Multi-Label NN | EPP-HMCNF | No-Share RF | Hierarchical Share RF | Multi-Label NN | EPP-HMCNF | No-Share RF | Hierarchical Share RF | Multi-Label NN | EPP-HMCNF |
| Mean score across ECs | No inhibitors 0.940 0.928 0.969 0.965 | 0.312 0.251 0.381 0.402 | 0.274 0.230 0.334 0.351 | With inhibitors 0.937 0.931 0.967 0.973 | 0.310 0.264 0.384 0.414 | 0.267 0.241 0.335 0.359 | % Difference -0.32 0.32 -0.21 0.83 -0.64 5.18 0.79 2.99 -2.55 4.78 0.30 2.28 |
| % ECs for which technique is better No inhibitors 49 45 52 41 | 50 45 49 43 | 51 48 50 47 | With inhibitors 51 55 48 59 | 50 55 51 57 | 49 52 50 53 |

Note: (A) Under random data split on the full test set, (B) under random split on the reduced dataset (i.e. only including ECs for which models could be trained under the realistic split) and (C) under realistic split. Scores in bold indicate that the model trained with inhibitors has better performance for that metric.

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![Fig. 5. Results of training with inhibitor information. (A) Evaluation on the unlabeled test set and (B) evaluation on the inhibitor test set](https://academic.oup.com/bioinformatics/article/37/14/2017/6124277)

All scores are lower on the Inhibitor Test Set than on the Unlabeled Test Set, indicating that it is harder for the models to distinguish between positives and inhibitors than it is to distinguish between positives and unlabeled. The higher standard deviation also shows that the task of discerning inhibitors from positives is harder for some EC Numbers than for others. This is further confirmation that the inhibitors can be designated as ‘hard’ negative examples. However, EPP-HMCNF trained with inhibitors is the best model at discerning inhibitors from positives (Fig. 5B), confirming EPP-HMCNF as our model of choice.

#### 3.3 Realistic split

We analyzed our models based on a ‘realistic data split’ that contain potentially novel query compounds in comparison to the training dataset (Martin et al., 2017). We clustered the molecules in our dataset on the molecular fingerprints with an average cluster size of 10. We used UPGMA (the unweighted pair group method with arithmetic mean), a simple bottom-up hierarchical clustering method (Sokal et al., 1958). We saved molecules from singletons and smaller clusters, up to 20% of the total molecules, for testing. This split reduced the similarity between test and training molecules when compared to the similarity obtained under a random split (Fig. 6A and B). The realistic split affects the distribution of positive data in the training and testing sets. Figure 6C and D plots the distribution of the ratio $r = (\text{positives}_{\text{test} \cup \text{train}})/\text{positives}_{\text{train}}$ per EC Number under each splitting strategy. Under the random split, $r$ appears to have a unimodal, symmetric distribution centered at 20%. Under the realistic split, $r$ follows an asymmetric distribution concentrated near 0% and decaying at larger values, similar to an exponential distribution. This distribution’s shape results in high number of EC Numbers with either no positive test data, or no or little training data.

Under the realistic split, models could be trained only for 680 EC Numbers, with an EC hierarchy composed of 6 Classes, 45 Subclasses and 122 Sub-subclasses. The relative performance of the models under the random split on this reduced dataset (Table 1B) is consistent with the full test set (Table 1A) across all metrics and in terms of average ranking per method. We compare the performance on this reduced dataset under the two different data splits (Table 1B and C). The relative performance of all models is lower under the realistic split than under the random split, with models trained with inhibitor data demonstrating slightly less degradation in mean AP and mean R-PREC. These results show that it is more difficult to obtain good model performance under the more challenging realistic split. Importantly, mean scores for almost all metrics are improved when training with inhibitors, with Multi-Label NN also experiencing a consistent boost. Crucially, EPP-HMCNF trained with inhibitor information is still the best model for the task (Mean R-PREC 0.126 versus 0.096 of $k$-NN Similarity).

#### 3.4 Predicting on natural versus non-natural query molecules

We evaluated the difference in performance of our best performing classifier, EPP-HMCNF trained with inhibitor information, on
enzyme classes acting on natural and non-natural substrates in the Full Test Set (under the random split). For both sets of predictions, we plotted (Fig. 7) the ratio of probabilistic predictions above a series of thresholds between 0 and 1. As all test instances in this experiment are positive, higher probabilistic predictions are desirable, which translate to a higher area under the curve (AUC). The predictions on the interactions with the natural substrates have higher AUC than the predictions on the interactions with the non-natural ones (0.846 versus 0.761). We restricted the comparison to EC numbers that had both natural and non-natural test compounds. For each EC Number, we computed the mean of the predictions on the natural versus non-natural test compounds. The AUC for the former is higher than the AUC of the latter (0.852 versus 0.761). These results suggest that it is easier for EPP-HMCNF to discern enzyme class interactions with natural substrates than with non-natural ones. Importantly, per Figure 7, the model still classifies over 61% of non-natural compounds as positive with a probability above 90%, and over 78% of non-natural as positive with probability above 50%. This illustrates the potential of machine learning models to identify new non-natural compounds, and thus promiscuous interactions, for our EC Numbers.

3.5 Speed and scalability of training and inference

We report training and inference speed of all models, each with their respective optimized set of hyperparameters, and trained for all 70 epochs where applicable (Table 2). The time complexity (discussed in Supplementary File S1, Supplementary Section S6) is also reported. All measurements are made when training the models on the Training set and Full Test set generated via random split. Models were trained and evaluated on an Intel(R) Xeon(R) CPU X5675 with a speed of 3.07 GHz and 64 GB RAM. Further speed-ups can be obtained via training on GPUs. K-NN similarity is expensive during inference, whereas, for all our dataset of 1659 molecules, all machine learning-based models ran under 1.5 min, generating predictions for 983 EC Numbers. The fastest models at inference time are the NN-based models. Their runtime is independent of the number of EC Numbers of interest as the output layer calculates all predictions simultaneously, thus making them appealing for large and comprehensive sets of predictions. As more data becomes available, Multi-Label NN and EPP-HMCNF hold promise to be the fastest and most scalable inference models.

4 Conclusion and discussion

This work proposed and evaluated several machine learning models to predict enzyme promiscuity on a query molecule. Our results show that sharing information both horizontally across enzymes and vertically across the EC hierarchy results in large and consistent gains in prediction quality. EPP-HMCNF implementation trained with inhibitor information is the best model for the enzyme promiscuity prediction task, achieving a Mean R-PREC of 0.359 across 983 EC Numbers, against the 0.265 Mean R-PREC of k-NN Similarity. For a typical enzyme, slightly more than 1 in 3 wet-lab trials would succeed if we selected the top-ranked molecules using EPP-HMCNF, while a similarity-based method would yield success for 1 in 4 molecules. Thus, although similarity is a competitive baseline, it is outperformed by machine learning methods. Further, similarity is less consistent than EPP-HMCNF across EC Classes. Our data analysis and experiments show that inhibitors are hard negative examples. Indeed, EPP-HMCNF trained with inhibitor information performs with a Mean R-PREC of 0.955 on the Unlabeled Test set, while yielding a Mean R-PREC of 0.873 on the Inhibitor Test Set, indicating that inhibitors are harder to distinguish from positives when compared to unlabeled molecules. Furthermore, EPP-HMCNF trained with inhibitor information is the best model at discerning known positives from known inhibitors.

Table 2. Training and inference times reported in hh:mm:ss, and time complexity on training and inference

| Model                  | Train. Time | Infer. Time | Time complexity on training | Time complexity on inference |
|------------------------|-------------|-------------|-----------------------------|-----------------------------|
| k-NN Similarity        | 00:00:00    | 00:12:54    | No training                 | O(ec<sub>n</sub>, m)         |
| No-Share RF            | 00:12:36    | 00:00:21    | O(ec<sub>m</sub>, log(n))   | O(ec<sub>m</sub>, m D)       |
| Hierar-chical RF       | 00:20:48    | 00:01:22    | O(ec<sub>n</sub>, log(n))   | O(ec<sub>m</sub>, m D)       |
| Multi-Label NN         | 04:06:10    | 00:00:03    | O(n ec<sub>l</sub>)         | O(m ec<sub>i</sub>)          |
| EPP-HMCNF              | 09:38:40    | 00:00:06    | O(n ec<sub>n</sub>)         | O(m ec<sub>n</sub>)          |

Note: n, number of training molecules; m, number of inference molecules, D, maximum tree depth across all random forests; p<sub>m</sub>, number of training positive molecules for a specific EC Number; p<sub>n</sub>, average number of training positive molecules across EC numbers; ec<sub>i</sub>, number of EC Numbers on which the model is trained; ec<sub>n</sub>, number of nodes in the EC hierarchy used in training (note: ec<sub>i</sub> < ec<sub>n</sub> ≤ 4ec<sub>n</sub>/3; ec<sub>n</sub>, number of EC Numbers for which inference is performed (note: ec<sub>n</sub> ≤ ec<sub>i</sub>).

Fig. 6. Data distributions under random versus realistic split. (A) Histogram of maximum similarity between one test compound and all training compounds under random split. (B) Maximum similarity between one test compound and all training compounds under realistic split. (C) Distribution of r, the ratio of number of positive examples in test set over the number of positive examples in the training set per EC Number, under random split. (D) Distribution of r under realistic split.
The realistic data split generates a more challenging test set than when using the traditional random split, allowing more ‘realistic’ assessment of how well these models are likely to generalize to compounds which are different from those in the training set. EPP-HMCNF trained with inhibitor information remains the best model even on this harder task, with a mean R-PREC across 680 EC Numbers of 0.126. Thus, we expect a 1 in 8 wet lab hit rate, in contrast with the 1 in 10 hit rate provided by similarity. While it is important to evaluate models under circumstances that most closely mimic practical scenarios, splitting the data in a non-uniform way inevitably leads to per-label biasing under a multi-label setting, which may bias the models in making more accurate predictions for some labels over others. Future work should further address this issue. Overall, our results suggest that predicting enzyme promiscuity through machine learning techniques that leverage existing knowledge in databases hold promise to advance biological engineering practices. The work presented here can be improved by integrating alternative methods from the PU learning literature (Bekker and Davis, 2023; Zhang and Zuo, 2008) and by considering learned representations that better capture molecular structure than binary fingerprint vectors, e.g., (Jin et al., 2018).

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