Substrate
Substrate preparation

7. Background

The way the substrate is prepared (e.g. boiling, emulsifying, resting time, storage stability) will have different influence on different enzymes.

It is important when setting up a harmonised method, to minimize the sources of variability. Macromolecular substrates typically change their molecular structure when heated up in the presence of water. This could influence the way different enzymes react on these substrates.

8. Illustration

8.1. From laboratory experiment #1

8.1.1. Purpose

Assess the influence of starch heating time on the activity of 3 α-amylases (3.2.1.1).

8.1.2. Enzyme sources

Microorganisms from bacterial and fungal origins.

8.1.3. Experimental setup

Three amylases (A, B, C) were incubated with 5 starch preparations (Substrate prep. 1-5). The substrates were prepared according to the following table:

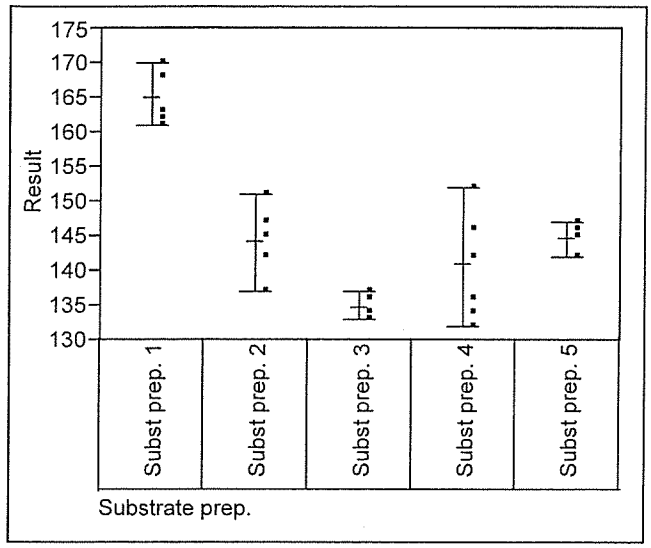
Substrate preparation	Preparation method
Substrate prep. 1	The substrate solution was heated up to 90°C – and then left to rest before analysis
Substrate prep. 2	The substrate solution was heated up to 95°C – and then left to rest before analysis
Substrate prep. 3	The substrate solution was heated up to the boiling point and boiled for 30 seconds – and then left to rest before analysis
Substrate prep. 4	The substrate solution was heated up to the boiling point and boiled for 5 minutes – and then left to rest before analysis
Substrate prep. 5	The substrate solution was heated up to the boiling point and boiled for 10 minutes – and then left to rest before analysis

Eight weighings of each product were measured with each substrate preparation of starch.

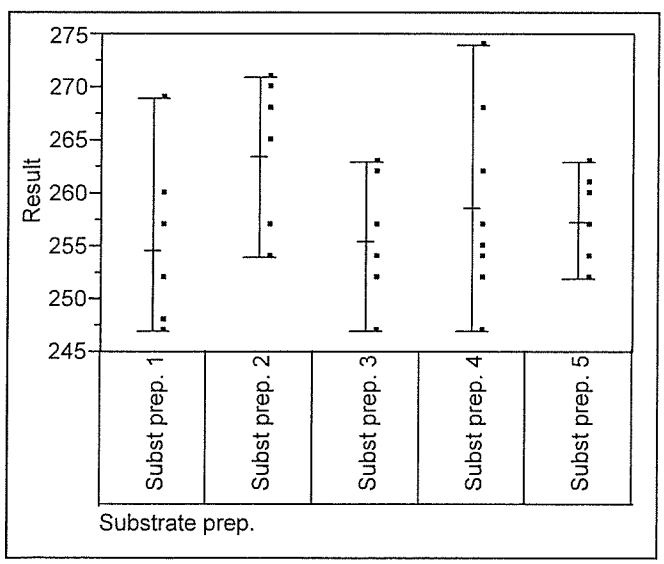
8.1.4. Results

The results are illustrated graphically below:

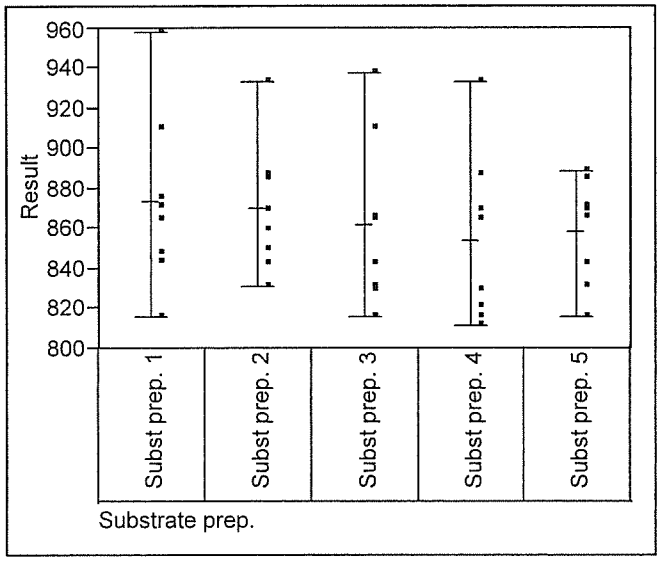
Sample: Amylase A
Variability Gage
Variability Chart for Result



Sample: Amylase B
Variability Gage
Variability Chart for Result



Sample: Amylase C
Variability Gage
Variability Chart for Result



Different mean values were obtained with Amylase A. For Amylase B and C no significant difference is observed.

Therefore, Amylase A is dependent on the substrate preparation method (boiling time). Amylase B and C does not show any dependency of the boiling time of the substrate.

8.2. From laboratory experiment #2

8.2.1. Purpose

Assess the influence of starch resting time on the activity of 3 α-amylases (3.2.1.1).

8.2.2. Enzyme sources

Microorganisms from bacterial and fungal origins.

8.2.3. Experimental setup

Three amylases (A, B, C) were incubated with 3 starch preparations (Substrate prep. 1-3). The substrates were prepared according to the following table:

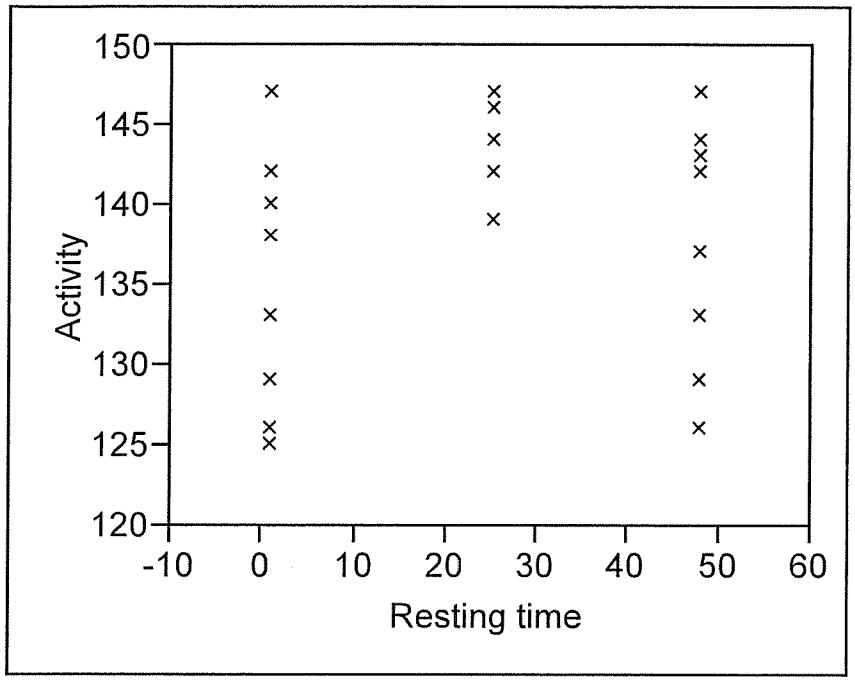
Substrate preparation	**Preparation method**
Substrate prep. 1	The substrate solution was heated up to the boiling point and boiled for 30 seconds – and then left to rest for 1 hour before analysis
Substrate prep. 2	The substrate solution was heated up to the boiling point and boiled for 30 seconds – and then left to rest for 24 hours before analysis
Substrate prep. 3	The substrate solution was heated up to the boiling point and boiled for 30 seconds – and then left to rest for 48 hours before analysis

Ten weighings of each product were measured with each substrate preparation of starch.

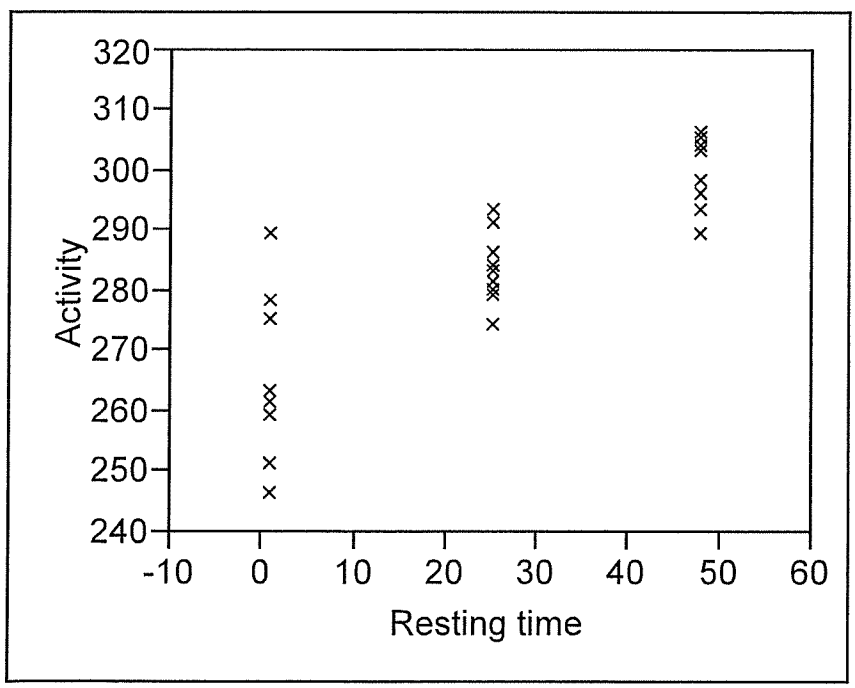
8.2.4. Results

The results are illustrated graphically below:

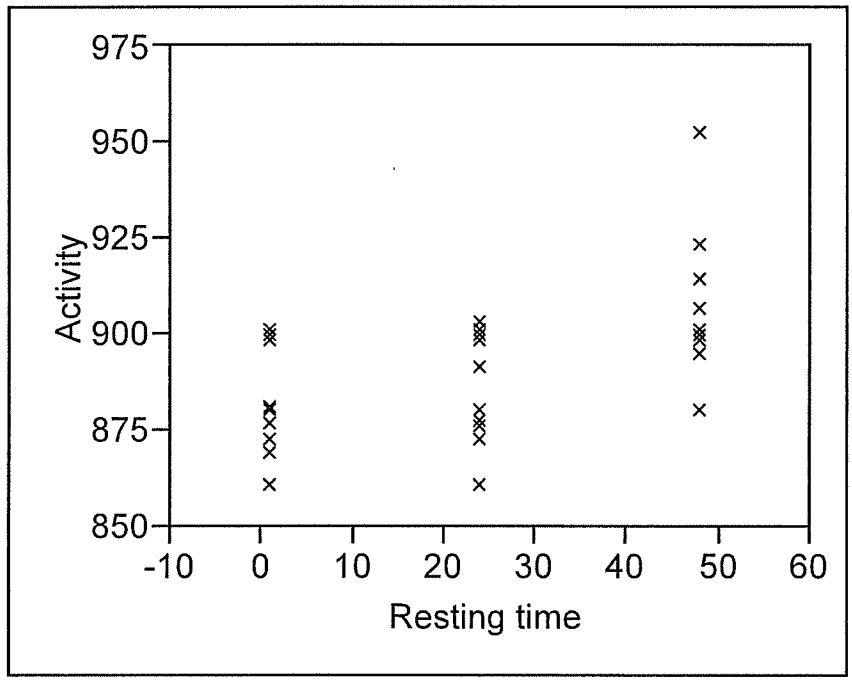
Product: Amylase A
Overlay Plot



Product: Amylase B
Overlay Plot



Product: Amylase C
Overlay Plot



Amylase A provides almost the same average results for the three substrate preparation methods. However the variation is different from substrate preparation 1 and 3 to substrate preparation 2. For Amylase B and C the activity seems to rise as the resting time go up.

It was tested if a reagent blank of 1, 24 and 48 hours would give response. No response was observed after 8 hours of reaction.

Therefore, Amylase A, B and C exhibit different activity patterns toward different resting times of the substrate.

9. Conclusion

It has been shown, using α-amylases as an example, that substrate preparation may in some cases have a definite influence on the assay result.

Assay conditions
Ions, buffers

10. Background

The presence and concentration of inhibitors or activators (e.g. metal ions) in the reaction mixture will have different effects on various enzymes.

A harmonised method would deal with different enzymes, whose sensitivity to a particular activator or inhibitor would vary across enzyme sources. It would then be necessary to ensure that the assay conditions, including the choice of buffers, make this influence negligible.

11. Illustration

11.1. From literature source #1

Properties of lactase produced by *Candida pseudotropicalis*. Castillo F.J. & Moreno, B. *Journal of Dairy Science 66(8), 1616-1621* (1983).

Enzyme under consideration: lactase (3.2.1.23).

The following figure shows the activation effect of the Mg^{2+} and Mn^{2+} ions on the enzyme activity:

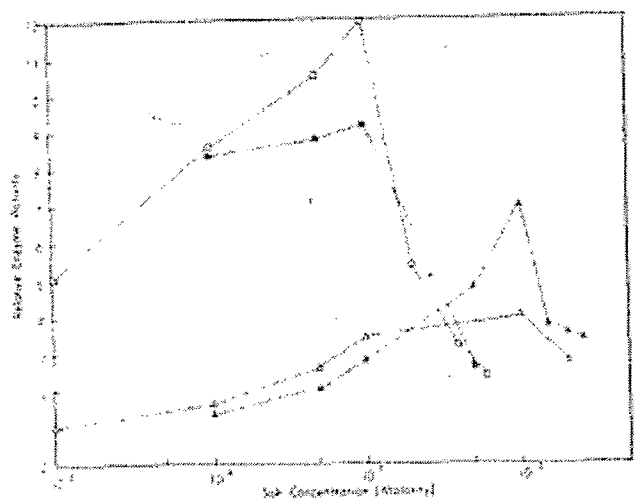


Figure 3. Activation of lactase activity by Mn^{++} and Mg^{++} salts. The enzyme was dialyzed against distilled water for 24 h and the salts added at the concentrations shown. The relative enzyme activity shown corresponds to the ratio: activity in the presence/absence of added salt. $MnCl_2$ (○), $MnSO_4$ (●), $MgCl_2$ (△), $MgSO_4$ (▲).

The following figure shows the inhibition effect of several ions on the enzyme activity:

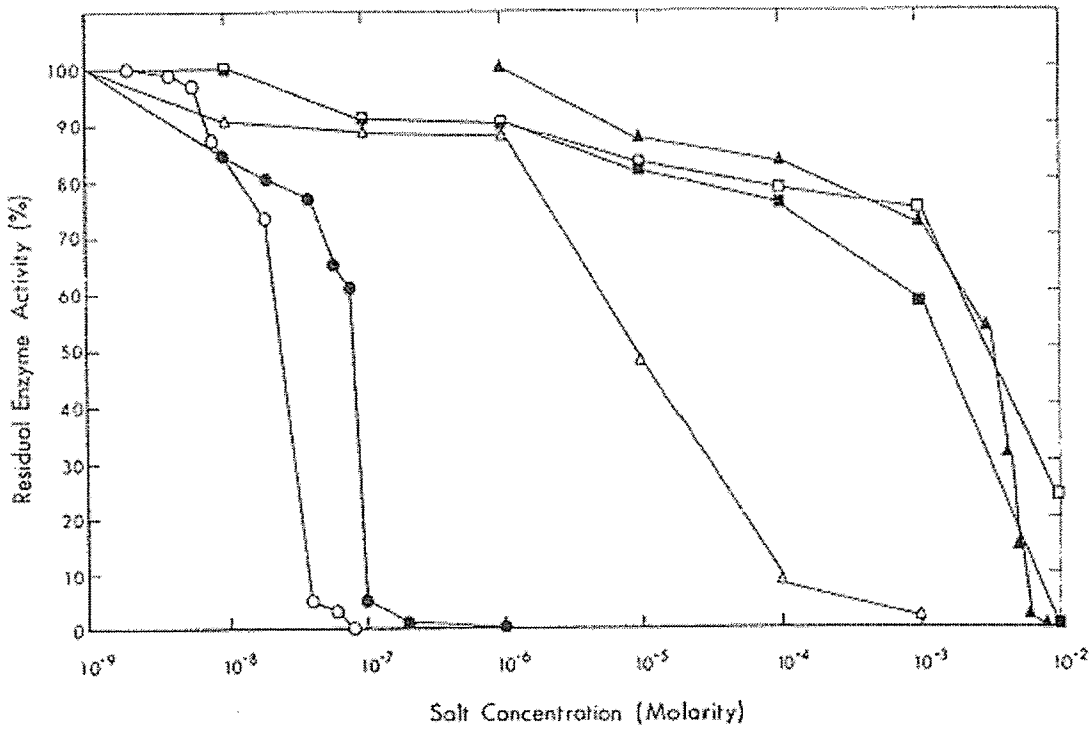


Figure 4. Inactivation of lactase activity by different cations. $HgCl_2$ (○), $AgNO_3$ (●), $CuSO_4$ (△), $ZnCl_2$ (▲), $CaCl_2$ (□), $CdCl_2$ (■).

11.2. From literature source #2

Inhibition of potato phenol oxidase by anions and activity in various carboxylate buffers (pH 4.8) at constant ionic strength. Malkin, B.D, Thickman, K.R., Markworth, C.J., Wilcox, D.E. & Kull, F.J. *J. Enzyme Inhibition 16, 135-145* (2002).

This paper illustrates the influence of various ions and buffers on the activity of phenol oxidase.

12. Conclusion

The assay conditions, in particular the concentration of any activating or inhibiting ion and the nature of the reaction buffer, should be adjusted so as not to be in a zone where the enzyme activity would vary greatly.

Substrate
Nature of the substrate

13. Background

A given enzyme may act on a variety of possible substrates. The choice of the substrate for the harmonised method will have different influence on different enzymes.

A harmonised method should use a substrate on which all enzymes give a significant response.

14. Illustration

14.1. From laboratory experiment #1

14.1.1. Purpose

Investigate the influence of substrate on the activity of several xylanases (3.2.1.8).

14.1.2. Enzyme sources

Microorganisms from bacterial and fungal origins.

14.1.3. Experimental setup

Parameter	Value / description
pH	5.0
Reaction time	10 min
Temperature	40°C
Buffer	McIlvaine
Substrate	Variable parameter

Substrates used:

- Wheat arabino-xylan (wheat AX)
- Birch arabino-xylan (birch AX)
- Rye arabino-xylan (rye AX)
- Water-insoluble arabino-xylan (WIP)
- Water-soluble arabino-xylan (WSP)

Analytical method principles:

- WIP activity is determined as amount (weight) of solubilised xylan / g product relative to an enzyme standard.
- Colorimetric activity (not tabs) is determined as µmol xylose released / min / g product.

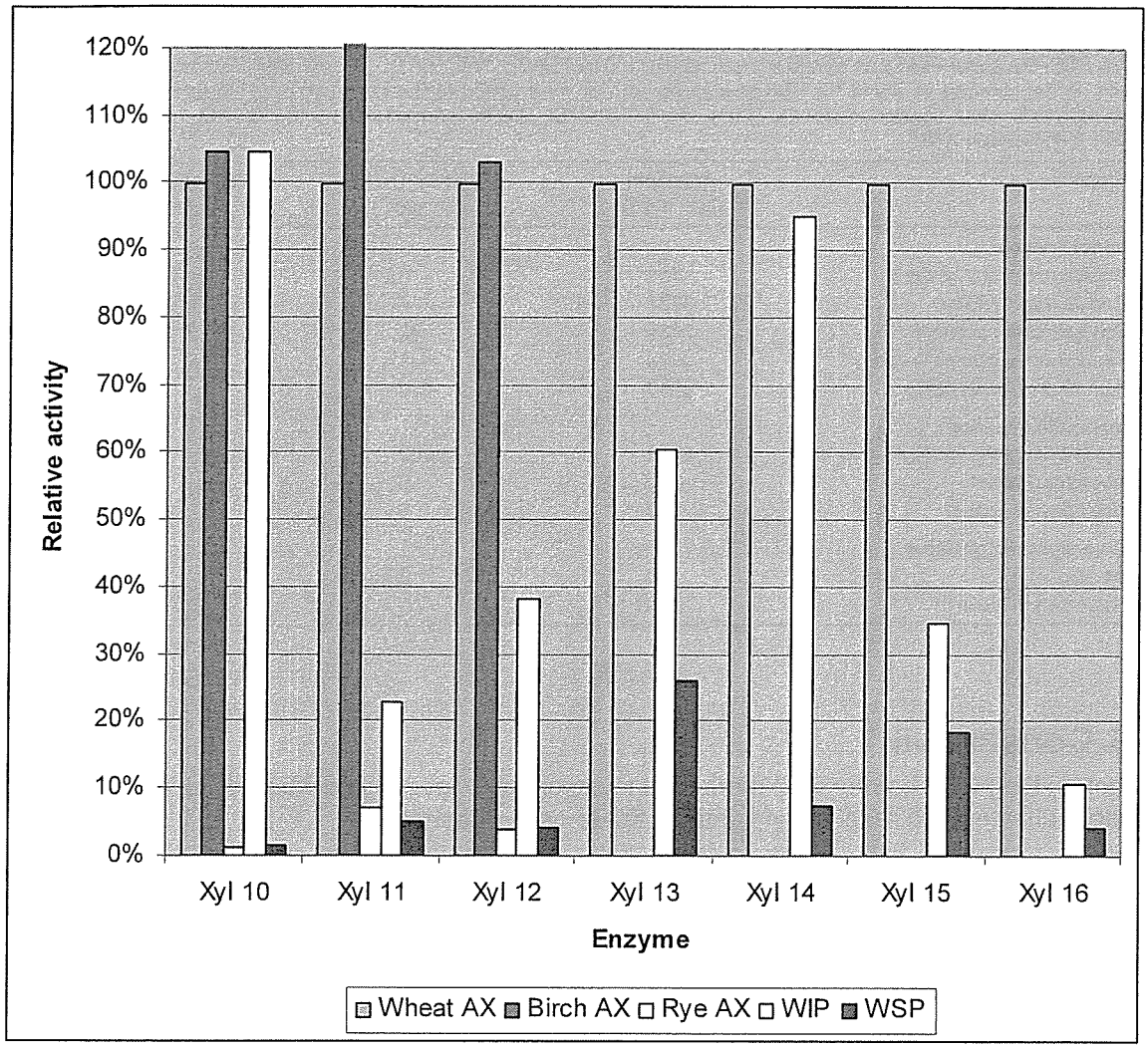
- Colorimetric activity (tabs) is determined relative to an enzyme standard.

14.1.4. Results

The following table shows the results of the determination of the activity of 7 xylanases, relative to the wheat arabino-xylan substrate:

Relative activities (relative to determinations on wheat AX tabs)					
	Wheat AX	Birch AX	Rye AX	WIP	WSP
Xyl 10	100%	105%	1%	105%	1%
Xyl 11	100%	198%	7%	23%	5%
Xyl 12	100%	103%	4%	38%	4%
Xyl 13	100%	-	-	60%	26%
Xyl 14	100%	-	-	95%	7%
Xyl 15	100%	-	-	35%	18%
Xyl 16	100%	-	-	11%	4%

The following figure shows the same results in graphic form:



Conclusion: it is clear that the source of the arabino-xylan substrate exerts an important and differential influence on the 7 xylanases under consideration.

14.2. From literature source #1

Methods for lipase detection and assay: a critical review. Beisson, F., Tiss, A., Rivière, C. & Verger, R. *Eur. J. Lipid C. Technol. 133-153* (2000).

The following table illustrates the variety of responses obtained when assaying lipases on 2 different substrates:

Tab. 1. Initial rates of hydrolyis of tributyrin emulsions and resorufin ester by various pure proteins. Tributyrin assay conditions: with all the proteins assayed except RGL, the buffer used was 1 mM Tris-HCl (pH 8), 150 mM NaCl, 10 mM $CaCl_2$. With RGL, the buffer used was 50 mM acetate (pH 6), 150 mM NaCl, 2 mM NaTDC, 1.5 µM BSA. Resorufin assay conditions (in the absence of *Thesit*™): 10 µl of a lipase sample were added to 90 µl of KH_2PO_4 0.1 M (pH 6.8) and 7 µl of resorufin ester stock solution in dioxane (1 mg · ml^{-1}). With RGL, the buffer used was 20 mM KH_2PO_4 (pH 6.8), 150 mM NaCl, 0.05% Triton X100.

Protein	Tributyrin [IU · mg^{-1}]	Resorufin ester (IU · mg^{-1})	Tributyrin/Resorufin ester ratio
Fungal lipases			
Candida antarctica lipase B	184	0.2	1022
Candida rugosa lipase	1037	214	4.8
Fusarium solani cutinase	3180	32	99
Pseudomonas glumae lipase	3000	401	7.5
Rhizomucor miehei lipase	8240	450	18.3
Mammalian lipases			
Human pancreatic lipase + Colipase	8000	1000	9
Lipoprotein lipase	250	8	31.2
Rabbit gastric lipase	800	3	267
Non enzymatic proteins			
Hemoglobin	0	0.7	0
Bovine serum albumin	0	0	–

15. Conclusion

When setting up a harmonised method, it is essential to investigate whether all enzymes under consideration react significantly to the chosen substrate.

Matrix effects
Presence of inhibitors in the test sample

16. Background

The test sample may contain inhibitors or activators which have varying effects on different enzymes.

17. Illustration

17.1. From laboratory experiment #1

17.1.1. Purposes

Investigate the differential effect of wheat xylanase inhibitor on several xylanases.

17.1.2. Enzyme sources

Microorganisms from bacterial and fungal origins.

17.1.3. Experimental setup

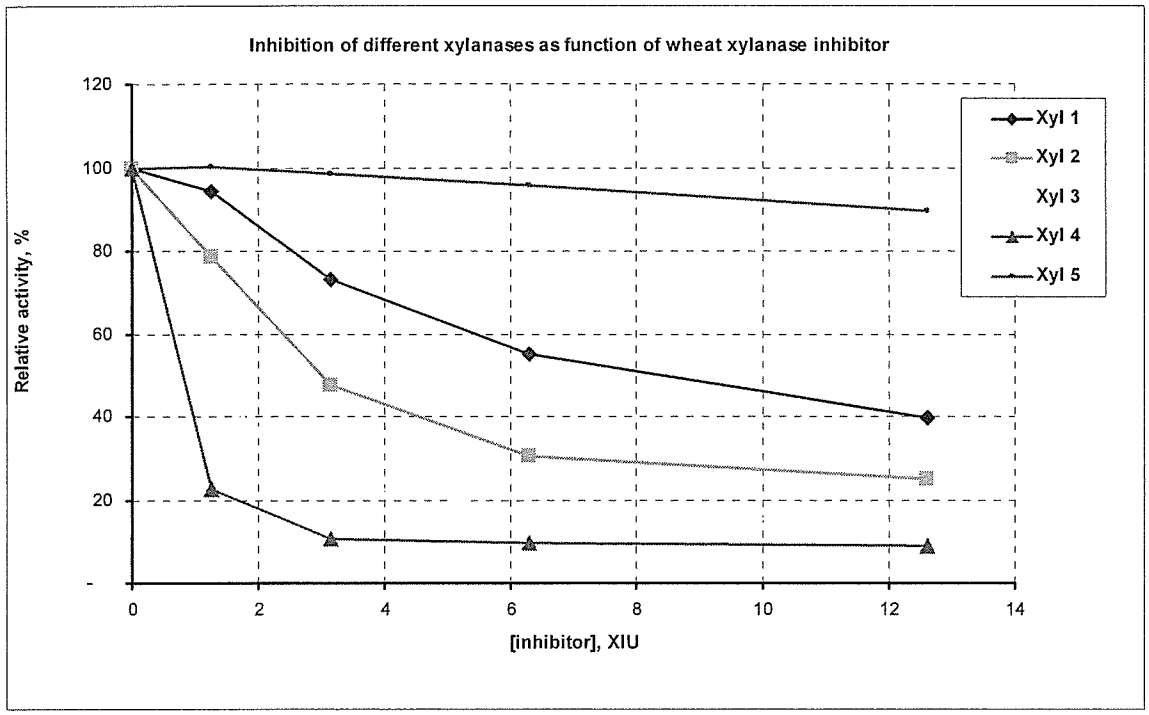
Parameter	Value / description
pH	5.0
Reaction time	10 min
Temperature	40°C
Buffer	McIlvaine
Substrate	Megazyme xylatabs (wheat based)

17.1.4. Results

Several inhibitor concentrations (expressed in xylanase inhibition units – XIU) were tested on 5 xylanases. The relative activities are shown below:

[inh], XIU	Xyl 1	Xyl 2	Xyl 3	Xyl 4	Xyl 5
0	100	100	100	100	100
1.26	94	79	45	23	100
3.15	73	48	24	11	99
6.3	55	31	17	9	96
12.6	40	25	14	9	90

In a graphic form:



17.1.5. Discussion

It is clear from the results that different xylanases show a very variable response to the presence of an inhibitor present in wheat.

18. Conclusion

When setting up a harmonised method for the measurement of enzyme activity in a complex matrix, it is necessary to investigate the effect of inhibiting or activating substances present in the matrix, which could affect various enzymes in a very different way.

Additionally, the amount of such inhibitors in matrices from vegetal origin is variable according to cultivar, harvesting conditions, climate, geographical origin (among other factors), as can be seen from e.g.:

Variations in the levels of different xylanase inhibitors in grain and flour of 20 French wheat cultivars. Bonnin, E., Daviet, S., Gebruers, K., Delcour, J.A., Goldson, A., Juge, N. & Saulnier, L. *Journal of cereal Science 41, 375-379* (2005).

Association of Manufacturers and Formulators of Enzyme Products

管理目的のための酵素活性の測定に関する Amfep の意見及び提案

補遺 2：統一測定法分析のためのパラメータ

測定条件
反応 pH

1. 背景

同じ EC 分類に属する酵素でも異なる生物的基原から得られると一般的に異なる至適反応 pH を示すことが普通に見られる。

また、既知酵素の一次構造(アミノ酸配列)を修飾すると、最適反応 pH の顕著な変化が一般的に見られる。

統一測定法でカバーされたグループ(群)に属する既知酵素の pH プロフィールが複数ある場合、どのように統一測定法の反応 pH を選べばよいだろうか？

未知又は性質が分かっていない酵素にはどう対処したらよいだろうか？

2. 実例

2.1. 試験室実験例＃1

2.1.1. 目的

EC 3.2.1.8（エンド-1,4-β-キシラナーゼ）に属する三つの酵素の pH 特性の確立

2.1.2. 酵素の基原

細菌とカビを基原とする微生物

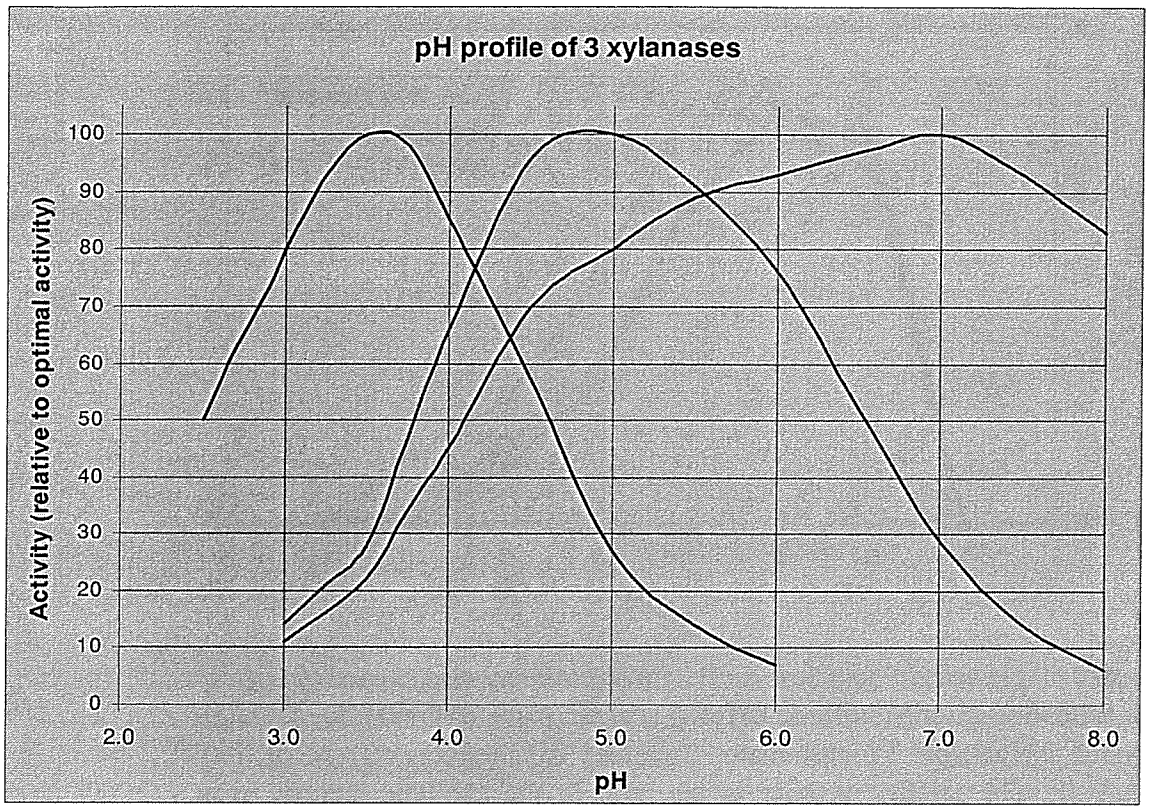
2.1.3. 実験方法

パラメータ	値／説明
pH	可変パラメータ
反応時間	１０分
温度	４０℃
緩衝液	マッキルベイン
基質	Megazyme xylatabs（小麦をベースにした）

2.1.4. 結果

各々の酵素の最高活性に対する相対活性で表示

	キシラナーゼ		
pH	#1	#2	#3
2.5	50		
2.7	63		
2.9	73		
3	80	14	11
3.25	93	21	16
3.5	100	28	22
3.75	98	47	34
4	85	66	45
4.5	57	96	70
5	27	100	80
5.5	14	91	89
6	7	76	93
6.5		52	97
7		29	100
7.5		14	93
8		6	83



2.1.5. 考察

検討した3種の酵素は、至適pHが3.5から7の間で顕著に異なるpH特性を示す。

検討した3種の酵素のうち2種は、急勾配のpH曲線を示す。このことは、反応液の少しの実験pHの誤差、バラツキが活性測定に著しい変動を引き起こすことを意味する。

2.2. 文献情報#1

Directed evolution of a bacterial α-amylase: Toward enhanced pH-performance and higher specific activity. Cornelius Bessler, Jutta Schmitt, Karl-Heinz Maurer, Rolf D. Schmid. *Protein Science 12, 2141–2149* (2003).
細菌αーアミラーゼ進化の方向：pH反応性の強化及びより高い比活性の方へ
Cornelius Bessler, Jutta Schmitt, Karl-Heinz Maurer, Rolf D. Schmid. *Protein Science 12, 2141–2149* (2003).

検討酵素：αーアミラーゼ(EC 3.2.1.1)
実験結果を下図に示す。

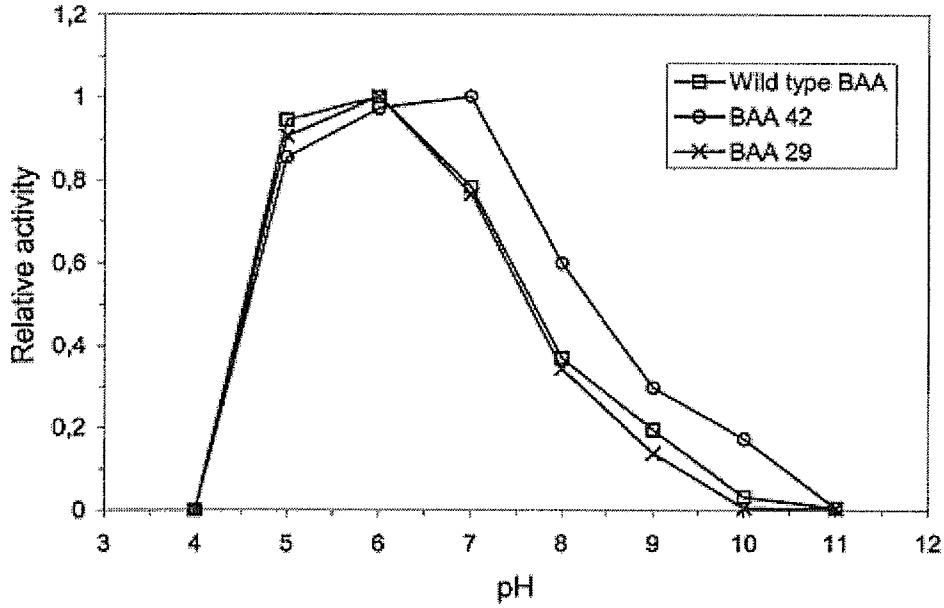


Figure 6. pH activity profiles. Activity measurement was carried out using the following 50-mM buffers: pH 4–6 acetate, pH 7–8 Tris/HCl, pH 9–10 glycine NaOH, pH 11 carbonate. (squares) WT, (circles) BAA 29, (×) BAA 42. The relative activity is the ratio of the catalytic activity at a certain pH and the maximum activity of each enzyme.

結論：蛋白質工学で酵素蛋白質を修飾すると、顕著な pH 特性の変化を引き起こす。

2.3. 文献情報＃2

A Novel, High Performance Enzyme for Starch Liquefaction. Discovery And Optimization Of A Low pH, Thermostable **a**-Amylase. Toby H. Richardson, Xuqiu Tan, Gerhard Frey, Walter Callen, Mark Cabell, David Lam, John Macomber, Jay M. Short, Dan E. Robertson, Carl Miller. *The Journal of Biological Chemistry 277(29), 26501–26507* (2002).
デンプン液化用の新規な高性能酵素－低 pH、熱安定なα－アミラーゼの発見と最適化
Toby H. Richardson, Xuqiu Tan, Gerhard Frey, Walter Callen, Mark Cabell, David Lam, John Macomber, Jay M. Short, Dan E. Robertson, Carl Miller. *The Journal of Biological Chemistry 277(29), 26501–26507* (2002).

検討酵素：α－アミラーゼ(EC 3.2.1.1)
実験結果を下図に示す。

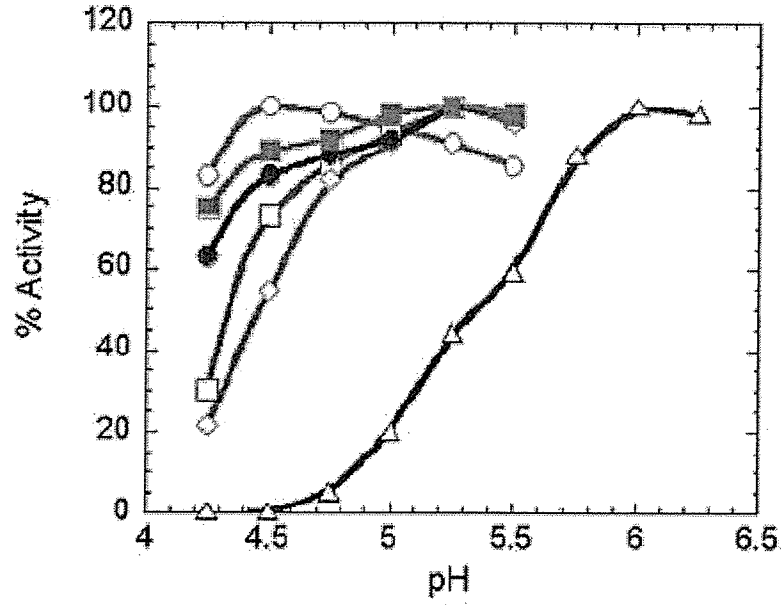


FIG. 2. **Determination of α-amylase pH optima.** The pH optima of wild-type and reassembled α-amylases were measured under typical industrial liquefaction conditions: 32% w/w starch slurry, 5 min treatment at 105 °C followed by 90 min at 95 °C. The dosage of amylase needed to achieve a target DE of 12 at the optimum for each α-amylase was determined. The same dosage was then used in liquefaction experiments at the remaining pH values, and the response was measured. The percentage of the maximal response at each pH is given as follows: △, *B. licheniformis* α-amylase; ◇, BD5031; □, BD5064; ●, BD5088; ○, BD5063; ■, BD5096.

結論：蛋白質工学で酵素蛋白質を修飾すると、顕著な pH 特性の変化を引き起こす。

3. 結論

統一測定法を設定することには、その方法によってカバーされる全ての酵素に適した反応 pH を選択するということが含まれる。

上記の実験結果は、次の酵素間では pH 特性及び至適 pH がかなり交差することを示している。

- 異なる生物学的基原に由来する酵素
- 同じ EC/IUB 分類に属する野生型の酵素と蛋白質工学で得られた酵素

特に：

- 至適 pH は 3 pH 単位以上異なることがある
- pH 特性は急勾配を示すことがある

実際のところ、統一測定法で選択された pH が分析しようとする全ての酵素の至適 pH に一致する訳ではない。特に設定ポイントが pH または温度曲線の急勾配な部分にあるときには、活性測定時の小さな pH 変化が非常に大きな測定結果の誤差を生ずることがある。

基質
基質のバッチ毎のバラツキ

4. 背景

基質、特に天然高分子系基質は品質、タイプ、供給面で変わりやすい。

統一測定法の実施のためには、基質はその測定法を使用するすべての試験室で同じように反応する必要がある。

もしも酵素反応速度が基質のバッチや供給者によって変わると、すべての試験室で同じバッチが使われていることを確認する必要があり、また、使いきる直前に新しいバッチで再度標準化が必要か確認しなければならない。

5. 実例

5.1. 試験室実験例＃1

5.1.1. 目的

3種のα-アミラーゼ(3.2.1.1)の活性における、デンプンサプライヤーとバッチの影響評価

5.1.2. 酵素の基原

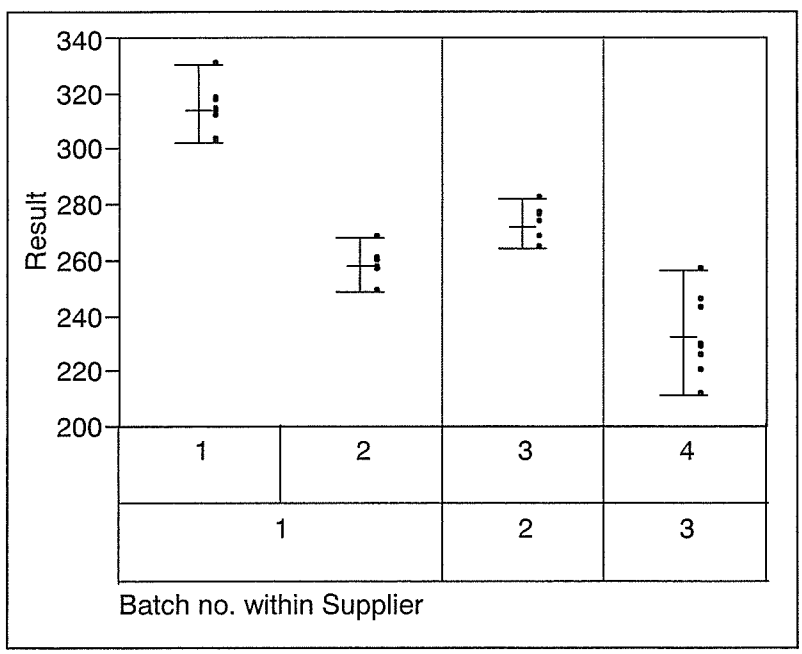
細菌とカビを基原とする微生物

5.1.3. 実験方法

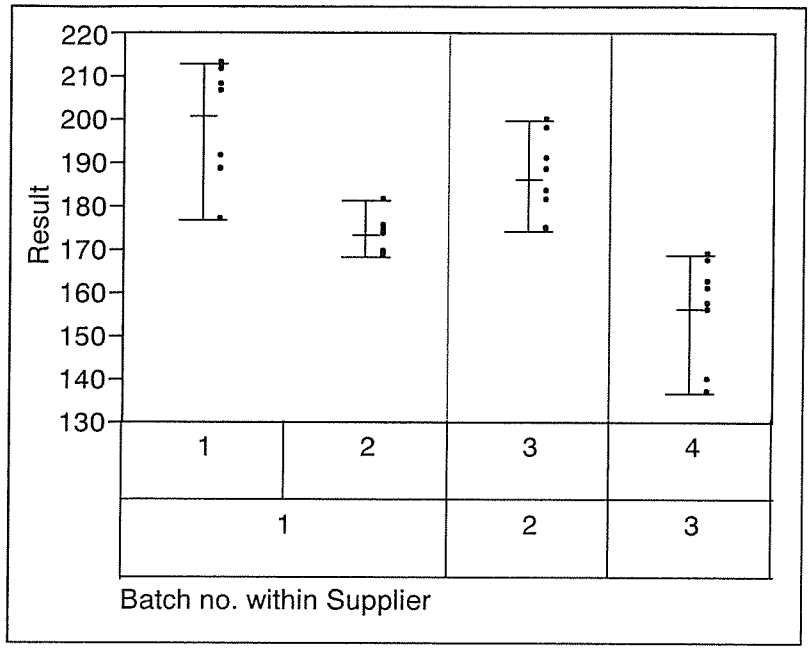
3種のアミラーゼ（A, B, C）と4つの異なるバッチ（バッチ1-4）のデンプンを反応した。バッチ1と2はサプライヤー1から、バッチ3と4はサプライヤー2と3からそれぞれ供給された。酵素活性はEB-SM-0009.02 version 1によって測定した（アミラーゼAとBはpH 5.6で、アミラーゼCは pH 4.7で反応)。それぞれの製品について8回秤量し、各バッチのデンプンを使用して測定した。

5.1.4. 結果

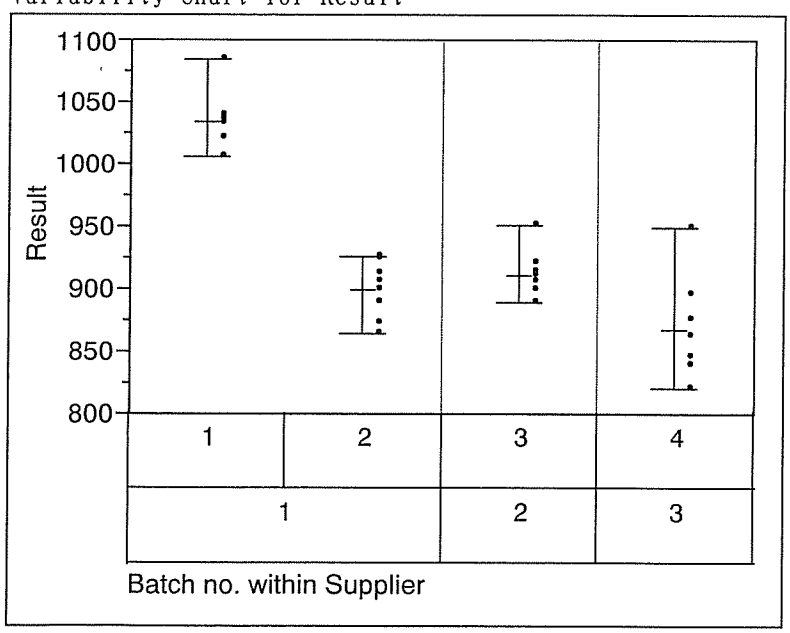
Sample: Amylase A
Variability Gage
Variability Chart for Result



Sample: Amylase B
Variability Gage
Variability Chart for Result



Sample: Amylase C
Variability Gage
Variability Chart for Result



5.1.5. 考察

デンプンのバッチが異なると、異なる平均値と異なる偏差が得られることがわかる。製品によってそのパターンは異なっている。アミラーゼCに関しては、基質バッチ2と3はほとんど同じ平均値であった。これはサプライヤーと基質がそれぞれ異なるものの、偶然に近似の値が得られたものと考えられる。

基質のバッチが重要であるか否か、次のSASjmpのモデルに従って確認した。
ln(Result) = μ + sample no. + batch no. + weighing + sample no.*batch no. + batch no.*weighing + sample no*weighing, Result was set to continuous, the rest to nominal.