From:
Sent: Wednesday, 20 February 2019 1:00 PM
To: standards management
Subject: FSANZ Submission Form Received (Internet) - Danisco Singapore Pte Ltd
Attachments: Technical information of Lipase 3 for application in cereal-based food and beverages.pdf

Application/Proposal Number:	A1159
Organisation Name:	Danisco Singapore Pte Ltd
Organisation Type:	Food Manufacturer
Representing:	DuPont Industrial Biosciences, DuPont Australia
Street Address:	
Postal Address:	
Contact Person:	
Contact Number:	
Email Address:	
Submission Text:	DuPont would like to request an amendment to the proposed use applications for the enzyme Triacylglycerol lipase from Trichoderma reesei, subject of A1159, to include “cereal based food and beverages” (retailed in both liquid and solid forms). This request would not involve any new usage of the enzyme, but gives food manufactures more flexibility in final format of

food produced, i.e. fermented vs non-fermented, liquid vs dried. As described in FSANZ Technical and safety assessment report of Application A1159, Triacylglycerol lipase in brewing assists to reduce lipid concentration and improve mash separation, specifically for non-malted cereals. The same function could be adopted by food manufactures out of brewing industry using malt and cereal as raw material. After mash separation, the wort (liquid) can either be fermented in subsequent steps to manufacture beer, or going through other manufacturing processes to make non-alcoholic drink. For example, the resultant process liquors (worts) can be evaporated for concentration into a malt syrup which can further be spray-dried to produce a malt flour. The malt extract can hereafter be used in production of cereal-based food and beverages. Regardless of the final format, the function and substrate of the Triacylglycerol lipase in the process remain the same as described in brewing process. Safety conclusion as described in A1159 in beer and brewing process would also apply to cereal based food and beverages. Therefore, DuPont consider the request a minor revision and hope FSANZ could consider accepting DuPont's request. Please refer to technical information attached for detailed justification for the revision.

Technical information of Lipase 3 for application in cereal-based food and beverages

Contents:

1 Identity

1.1 Lipase

The systematic name of the principle enzyme activity is triacylglycerol acylhydrolase. Other names used are lipase (*ambiguous*); butyrinase; tributyrinase; Tween hydrolase; steapsin; triacetinase; tributyrin esterase; Tweenase; amno *N*-AP; Takedo 1969-4-9; Meito MY 30; Tweenesterase; GA 56; capalase L; triglyceride hydrolase; triolein hydrolase; tween-hydrolyzing esterase; amano CE; cacordase; triglyceridase; triacylglycerol ester hydrolase; amano P; amano AP; PPL; glycerol-ester hydrolase; GEH; meito Sangyo OF lipase; hepatic lipase; lipazin; post-heparin plasma protamine-resistant lipase; salt-resistant post-heparin lipase; heparin releasable hepatic lipase; amano CES; amano B; tributyrase; triglyceride lipase; liver lipase; hepatic monoacylglycerol acyltransferase, Lipase 3.

The enzyme Lipase 3 is derived from *Trichoderma reesei* which is genetically modified to overexpress the Lipase 3 gene from *Aspergillus tubingensis*.

- EC number: 3.1.1.3
- CAS number: 9001-62-1

Please also refer to Appendix A of original submission.

1.2 Other enzymes

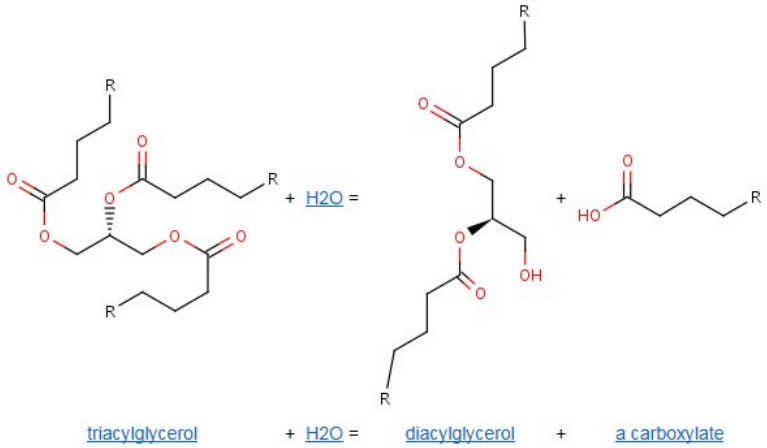
Downstream processing concentrates and purifies the enzyme product. The resulting enzyme preparation will not be totally pure and traces of other enzyme activities (e.g. protease) might be found but their level will be very low.

2 Chemical and physical properties

2.1 Substrate specificity

Triacylglycerol lipase (IUBMB 3.1.1.3) hydrolyses ester bonds primarily 1 and 3 position of fatty acids in triglycerides. Lipases belong to the carbohydrate esterase family 10. Carbohydrate esterase family 10 consists of various esterases acting on non-carbohydrate substrates (http://www.cazy.org (Cantarel *et al.*, 2009)) and sharing a common (α,ß/α) sandwich domain. Substrates include lipids (triglycerides).

Reaction catalyzed by triacylglycerol lipase (3.1.1.3), lipoprotein lipase (3.1.1.34), hormone-sensitive lipase (3.1.1.79)



triacylglycerol + H2O = diacylglycerol + a carboxylate

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2.2 Activity

The activity of the Lipase 3 is defined in LIPU (Lipase Unit (Tributyrin)). 1 LIPU (Lipase Unit (Tributyrin)) is defined as the amount of enzyme, which releases 1µmol H^+ per minute under the given condition. The assay is based on the Enzymatic hydrolysis of lipids releases fatty acids. For each fatty acid released, an equivalent H^+ appears. By continuous titration of H^+, the lipase activity can be determined based on the consumption of base. This method is not applicable for samples containing calcium chloride. $CaCl_2$ might interfere with certain lipases.

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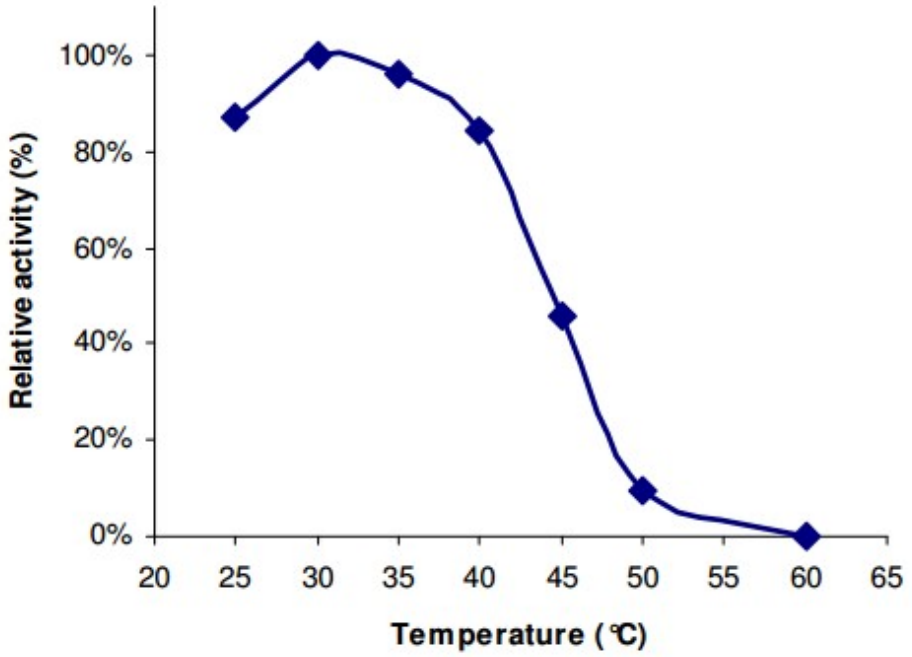
2.3 Temperature optimum

Enzyme activity of purified Lipase 3 ferment was determined at various temperatures. The method is based on pH-stat titration using an automatic Titralab Tim856 titration manager. The substrate was a homogenised emulsion of 5% (v/v) Glycerol tributyrate, 0.10% (w/v) Gum Arabic, 7.5% (w/w) Glycerol, 51 mM NaCl, 0.50 mM KH2PO4, pH 5.5 and the titrant was 0.05 M NaOH. When not otherwise mentioned, the reaction pH was 5.5 and the reaction temperature was 30°C. Results are shown in **Error! Reference source not found.**.

Temperature optimum was determined to be 30°C with relatively high relative activity up to 40°C. At 45°C and above, the relative activity is reduced to <50 %.

Figure 1: Temperature profile of Lipase 3 in the LIPU titration assay. Activity is shown as relative to the highest measured value.



Please also refer to Appendix A of original submission.

2.4 Thermal stability

Thermal stability of Lipase 3 was determined by measuring residual lipase activity after incubation at 70°C for 0-100 minutes in 50 mM Sodium acetate buffer, pH 5.5. The enzyme is inactivated after 100 min. incubation at 70°C.

Lipase 3 samples were diluted in sample buffer containing 50 mM Sodium Acetate, pH 5.5, 0.1% BSA, 1.2% NaCl, and incubated at 70°C for 0, 5, 10, 20, 30, 40, 60, 80, 100 min. The samples were stored on ice and the residual activity was then determined by the following method: The substrate was diluted in substrate solution containing 120 mM Sodium Acetate, pH 5.5, 0.2% Glycerol Trioctanoate, 0.3% NaCl, 13% Triton X-100. The assay sequence was 3 min equilibration of 34 ml substrate at 37°C before addition of 20 ml enzyme sample solution, and incubation for 30 min at 37°C before measurement of liberated free fatty acid. Blank measurements were performed by analysing samples without enzyme. Free fatty acid in the reaction mix was measured using the NEFA C kit (WAKO Chemicals, Neuss, Germany). 56 µl NEFA C reagent A was added to the enzyme reaction mix and incubated for 5 min at 37°C before addition of 113 µl NEFA C reagent B followed by incubation for 5 min at 37°C and, finally, OD520 was measured. The amount of liberated free fatty acid was calculated from an oleic acid standard curve with OD520 nm against mM FFA. 1 LIPU is defined as the quantity of enzyme that produces 1 microequivalent of free fatty acid per min under the conditions described. Results are shown in Figure 2.

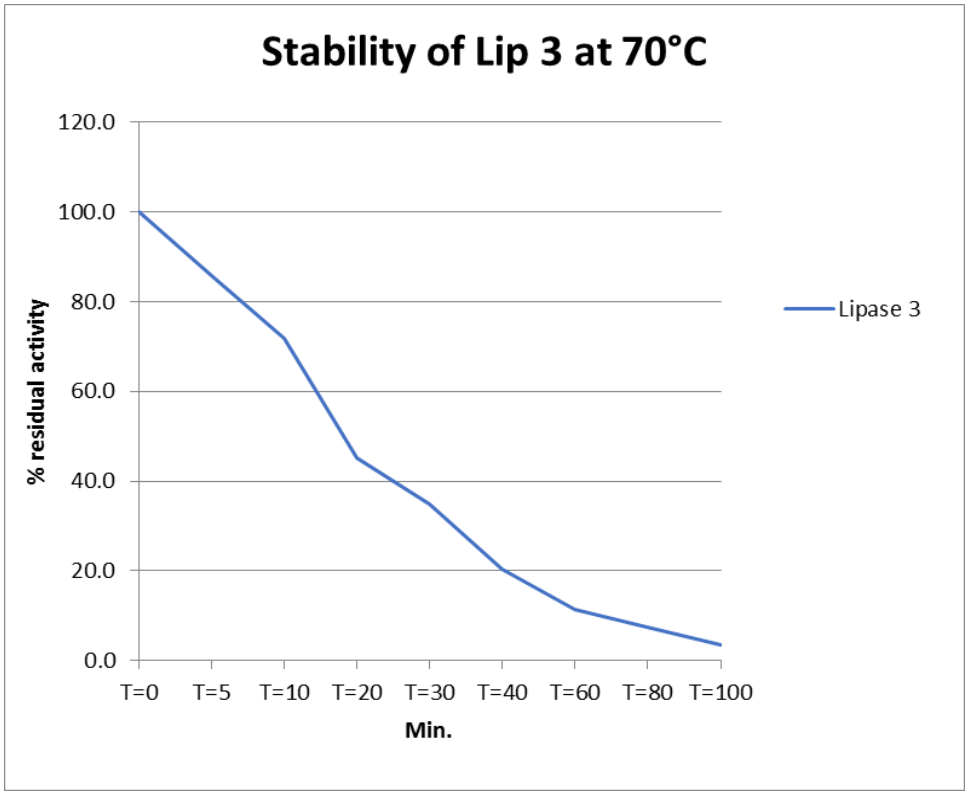


Figure 2: Activity was determined after 0-100 minutes of incubation in 50 mM Sodium acetate buffer, pH 5.5.

After 20 min incubation at 70°C, residual activity of Lipase 3 is 45 %. It is inactivated after 100 minutes of incubation at 70°C.

Please also refer to Appendix A of original submission.

2.5 pH optimum

Enzyme activity of spray purified Lipase 3 ferment was determined at various pH-values. For titration for pH profile, the reaction pH's were as stated in Figure 3. 2 ml enzyme sample was added to 25 ml substrate, pre-equilibrated at stated pH and temperature, before the titration was started. Activity was calculated from the slope of the titration curve with consumption of titrant against reaction time. 1 LIPU is defined as the quantity of enzyme that produces 1 microequivalent of free fatty acid per min under the conditions described. Results are shown in Figure 3.

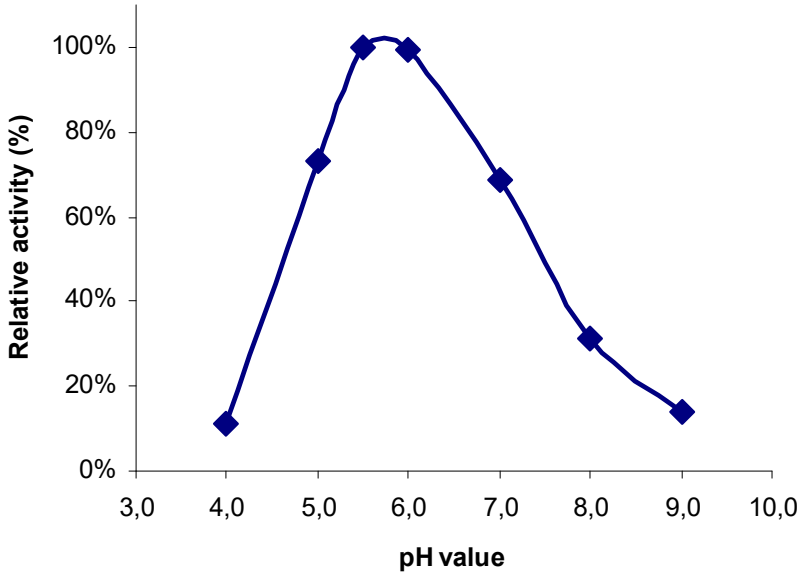


Figure 3: pH profile of Lipase 3 in the LIPU titration assay. Activity is shown as relative to the highest measured value

pH optimum is seen at pH 5.5-6 and the enzyme is active in the range from pH 4 to 9.

Please also refer to Appendix A of original submission.

2.6 Storage stability

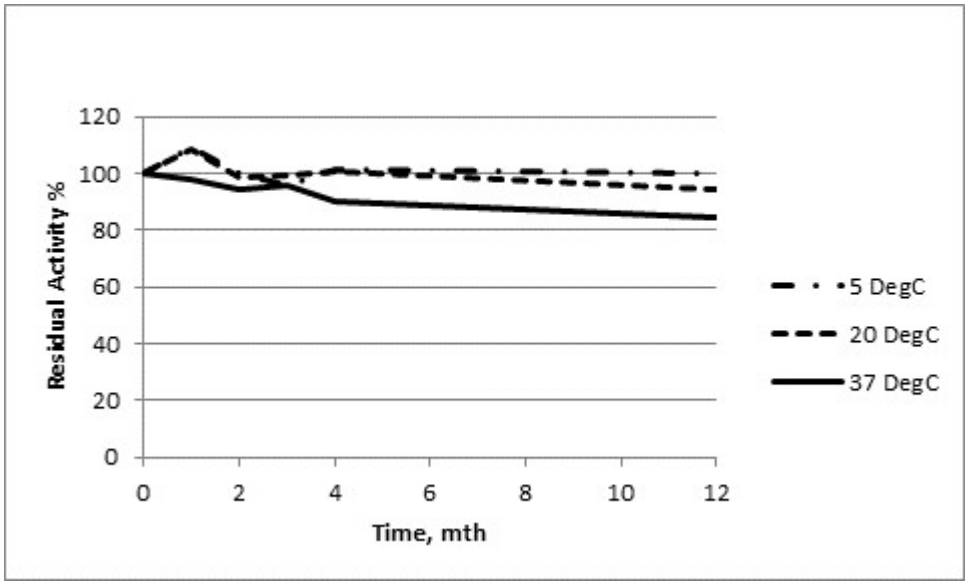


Figure 4. Storage stability of Lipase 3

As seen in the figure above, at 5°C and 20 °C, the enzyme is stable for more than 1 year without loss of activity.

Please also refer to Appendix A of original submission.

3 Efficacy and benefits of the Lipase 3 enzyme preparation

3.1 Description

As noted above, the function of Lipase 3 is to catalyse the hydrolysis of ester bonds primarily in 1 and 3 position of fatty acids in triglycerides with release of fatty acids and glycerol.

Cereal-based food and beverages

Lipase 3 can be added to the cereal-based food and beverage production to enhance the mash filtration in production of cereal extract. In the mashing step Lipase 3 hydrolyses triglycerides to enhance the mash filtration. The resultant process liquors (worts) are evaporation for concentration into a malt syrup which can further be spray-dried to produce a malt flour. The malt extract can hereafter be used in production of cereal-based food and beverages.

Therefore, the benefits of the conversion of lipids with the help of Lipase in Cereal-based beverages are:

- Faster and more predictable mash separation;
- Higher yield due to the improved processing, and thereby less use of raw materials.

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3.2 Efficacy examples

Cereal-based food and beverages

The function of Lipase 3 is to catalyse the hydrolysis of ester bonds primarily in 1 and 3 position of fatty acids in triglycerides with release of fatty acids and glycerol. Lipase 3 can be added to enhance the mash separation in production of cereal based food and beverages, primarily non-malted raw materials. In the mashing step Lipase 3 removes lipids to enhance the mash separation. Current enzyme solution (amylase, xylanase glucanase, protease, and pullulanase) using barley as raw material is functional up to 80% barley. Addition of Lipase 3 could help to achieve success with 100% barley as raw material.

In Figure 1, it can be seen that addition of Lipase 3 to existing enzyme mixture (amylase, xylanase glucanase, protease, and pullulanase) could improve wort filtration and reduce the haze of the wort, providing more clear wort using 100% barley as raw material. The increased filtration could be observed as early as 5 minutes. After thirty minutes of filtration, addition of Lipase 3 at 114 M LIPU/tn barley has approximately twice the volume of filtrate compared to the control.

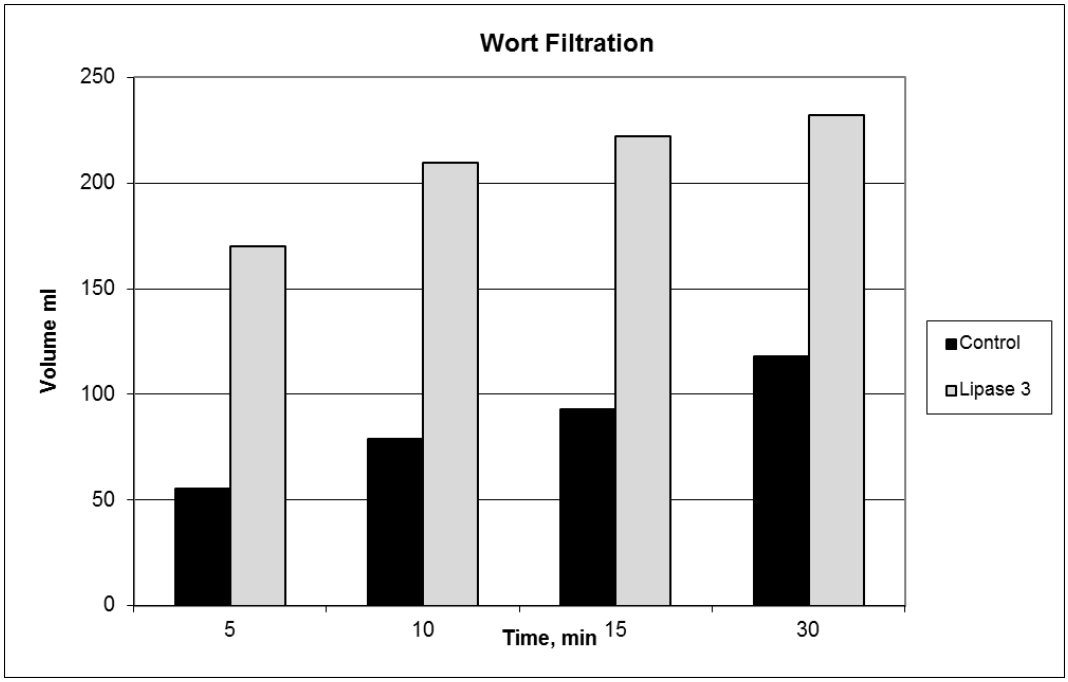

Figure 1. Adding lipase 3 improves wort filtration using 100% barley as raw material.

Control: Amylase, xylanase glucanase, protease, and pullulanase
Lipase 3: Lipase 3 + amylase, xylanase glucanase, protease, and pullulanase

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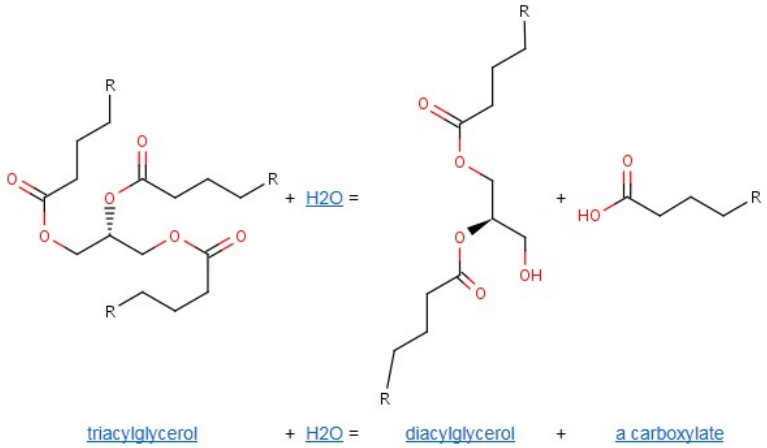

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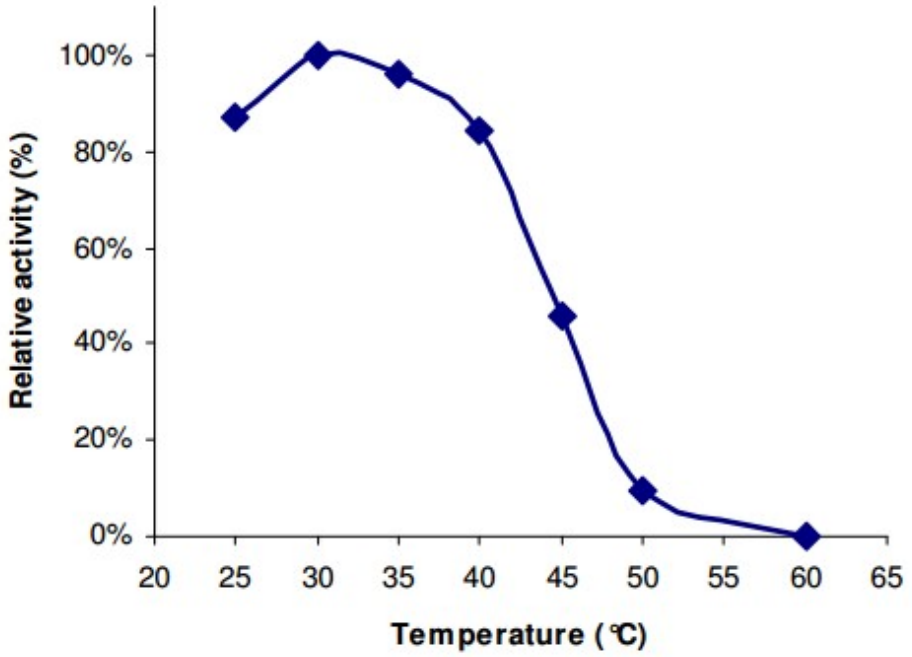

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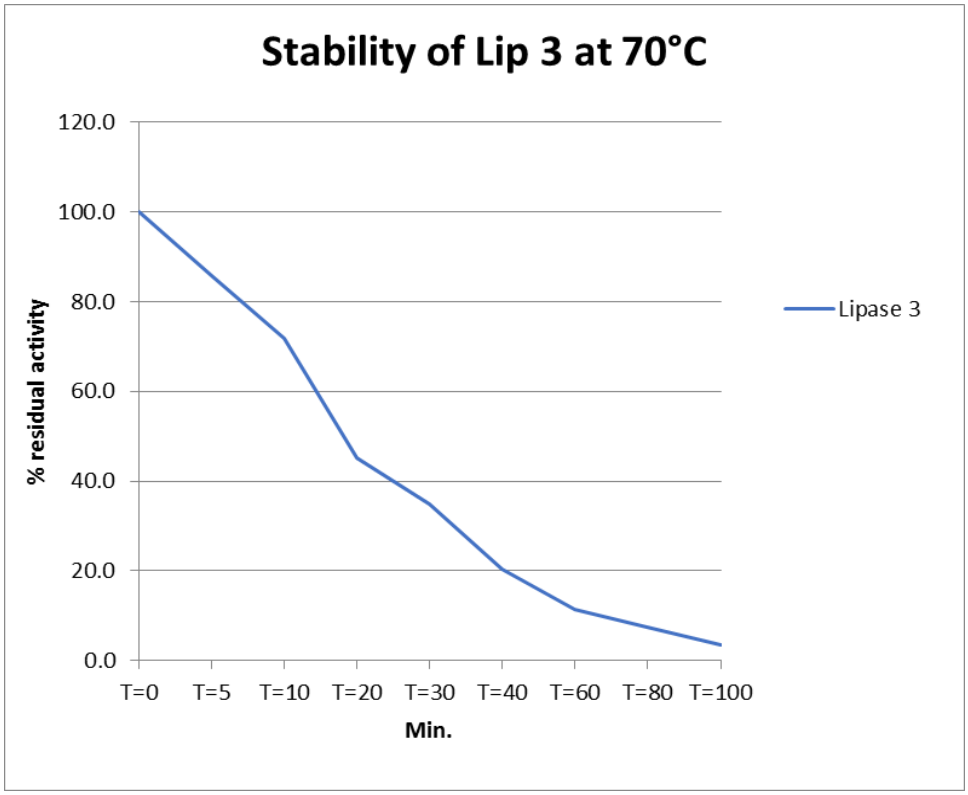

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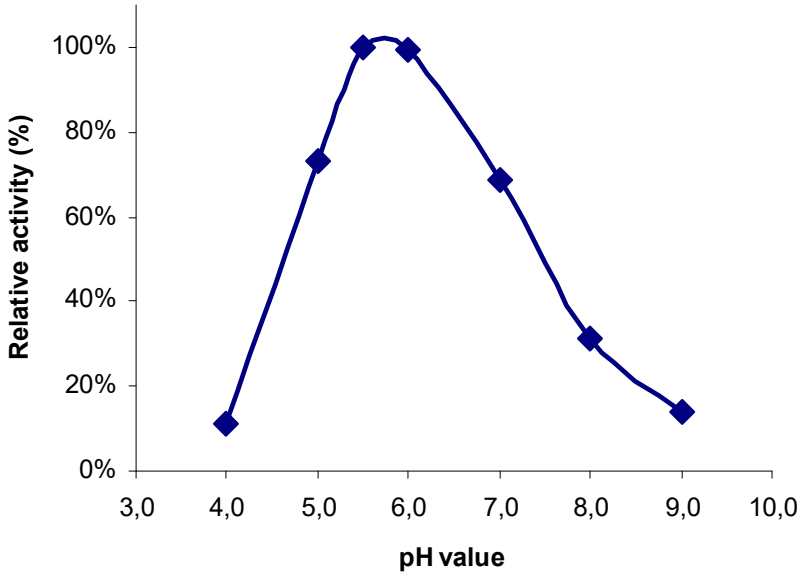

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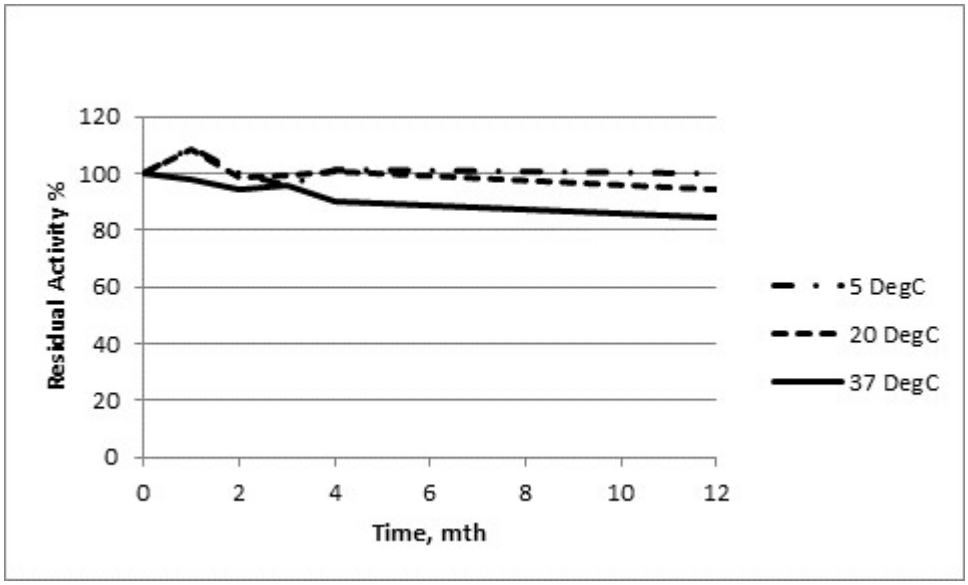

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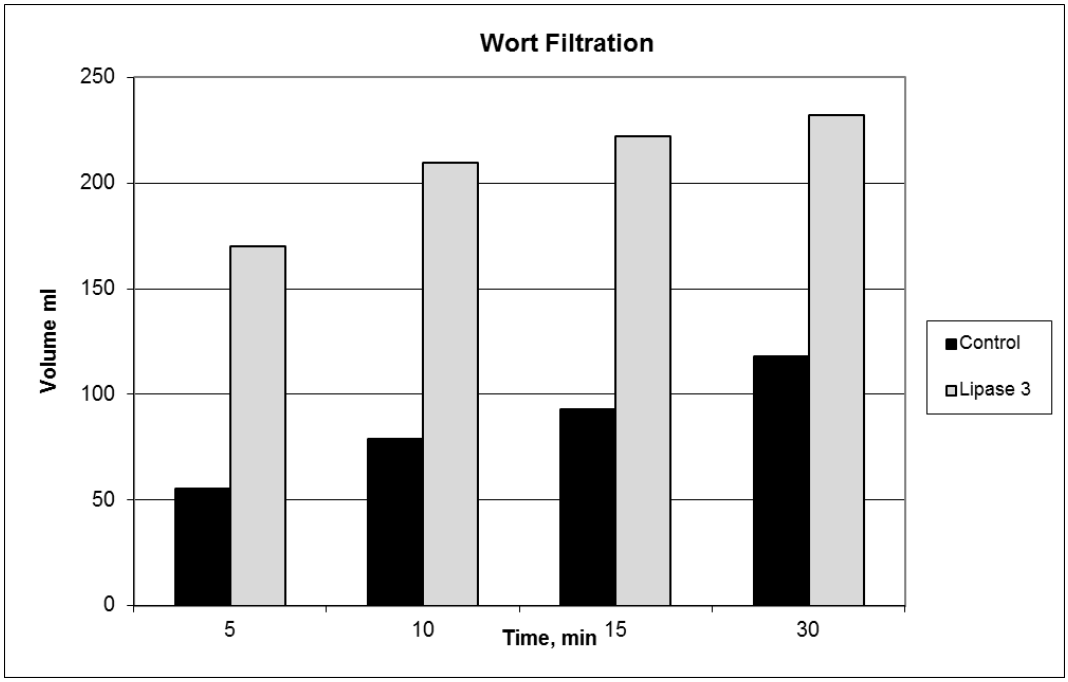

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