SUPPORTING INFORMATION

Effect of PEG-induced molecular crowding on the enzymatic activity and thermal stability of β galactosidase from *Kluyveromyces lactis*.

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Experimental conditions for β-Gal activity measurement in the presence of PEG_{6000}

a) Effect of PEG_{6000} on the spectroscopic characteristics of ONP.

Aqueous disolutions of *ortho*-nitro phenol (ONP) were prepared in the absence (PEG_0) or in the presence of PEG_{6000} at final concentrations ranging from 15 to 55 % w/v. The absorbance of the *ortho*-nitrophenoxide (ONPx) formed was determined, in a Beckman DU 700 (Fullerton, C. A.) spectrophotometer, at 420 nm (A_{420}) or within the range 260-500 nm. In some experiments a temporal variation of the absorbance at 377 nm (A_{377}) was registered.

b) Chemical stability of ONP in the presence of PEG_{6000}.

The hypothesis that PEG_{6000} could have induced changes on the spectral behaviour of ONP was explored more deeply. The absorbance spectra of ONP was analysed at two extreme pH. Only at basic pH (a condition that resembles that of the enzymatic study) the typical peak at 420 nm was shown due to the presence of the dissociated species (*o*-nitrophenoxide, ONPx) (Fig.1a). In the presence of PEG_{6000} and at an alkaline pH the maximum in the ONPx spectra exhibited a hyperchromic and bathochromic shift (Fig. 1b). Taking into account that the acid-base titration curves of ONP showed an increase in the intensity without a change in the position of the peak at 420 nm [1], it could be suggested that the presence of PEG_{6000} not only favours the displacement of the ONP acid-base equilibrium towards the dissociated species (ONPx) but also may modify the polarity of the solution.

A straight line was fitted to the absorbance at 420 nm vs. PEG concentration data (Fig.1c). After transforming A_{420} into molar extinction coefficients values (ε_{ONP}), the regression equation (eq.S1) allowed calculate ε_{ONP} at 420 nm at different PEG_{6000} concentrations which varied from 4,809.6 M^{-1} cm^{-1} at 0% w/v PEG_{6000} to 5,478.32 M^{-1} cm^{-1} at 45 % w/VvPEG_{6000}).

$$\varepsilon_0 = 4809.6\ M^{-1}cm^{-1} + 12.15 M^{-1}cm^{-1} \times [PEG_{6000}] \qquad [S1]$$

These values were used to estimate the correct amount of product obtained through the β-Gal enzymatic activity in the presence of PEG_{6000}.

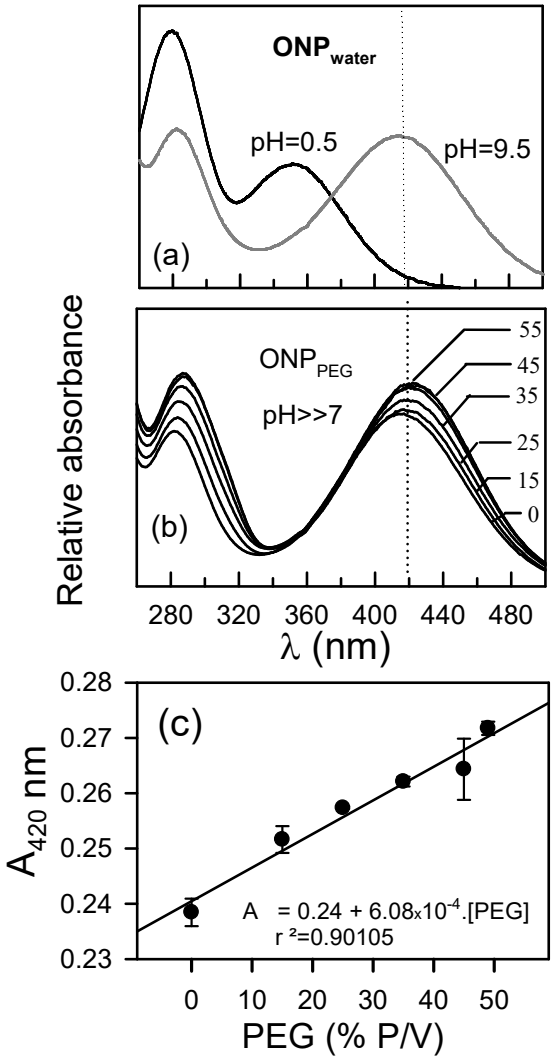

Fig.S1. Effect of PEG_{6000} on the UV-vis absorbance spectrum of ONPx.

Absorbance spectra of ONPx in water at two extreme pH (a) and at alkaline pH in solutions containing PEG_{6000} at the concentrations indicated (b).
In (c) a linear regression analysis of ONP absorbance at 420 nm vs. PEG concentration in an alkaline solution is shown. The regression equation is also depicted.

c) Testing different inactivators of β-Gal to stop the chemical reaction

In the present paper, β-Gal enzymatic activity in the absence and in the presence of different concentrations of PEG_{6000} was measured using ONPG as substrate. Usually, those enzymatic assays where ONP is the reaction product use Na_2CO_3 to stop the hydrolysis of the substrate (ONPG in the present case). At the same time, this procedure induces a sudden pH increase that displaces the ONP acid-base equilibrium towards the *ortho*-nitrophenoxide (ONPx), the dissociated form of ONP (Fig.1a). Different from ONP (λ_{max} ~ 352 nm in water), ONPx has an absorption peak within the visible region (λ_{max} ~ 420 nm in water) of the electromagnetic spectrum which allows its quantification based on spectrophotometric measurements avoiding the interference of other chemical species present in the reaction

mixture. However, in the presence of PEG_{6000}, Na_2CO_3 induces turbidity and a liquid-liquid phase separation, generating an interface where the enzyme and/or the product can be adsorbed [2-3]. Hence, instead of using Na_2CO_3, in the present paper we evaluated the efficacy of NaOH to inactivate the enzyme (Fig.2), after the incubation of ONPG in the presence of β-Gal, with or without 30% w/v PEG (PEG_{30} and PEG_0, respectively) and in optimal conditions for the enzyme activity (0.1 M sodium phosphate buffer pH 6.8 and 37 ºC). After the addition of NaOH, the absorbance of ONPx at 377 nm (A_{377}) was continuously recorded during 30 min to confirm the reaction arrest. Time zero was defined as the start of the inactivation procedure. Similar experiments were done using Na_2CO_3 as inactivator. The choice of 377 nm as the detection wavelength was due to the fact that this is an isosbestic point in the family of ONP spectra at different PEG concentrations (Fig.1b) (this reflect an independence of ONPx absorbance values on [PEG] at this λ).

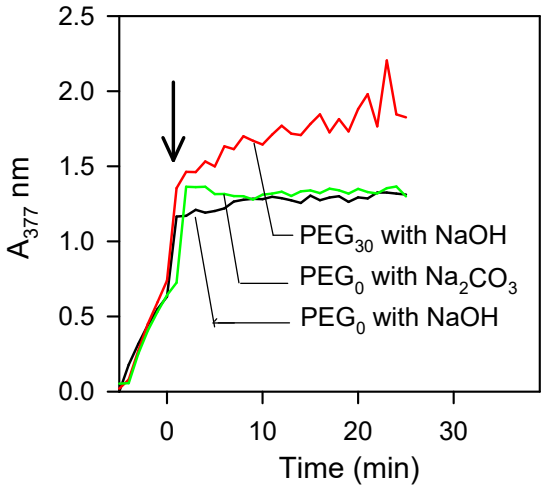

Fig. S2 Effect of the addition of inactivator on the β-Gal induced hydrolysis of ONPG.
The incubation system contained 8 mM [ONPG] and $1.27x10^{-3}$ mg/ml protein (β-Gal preparation) with or without 30% w/v PEG. After 10 min incubation at 37ºC, the inactivator (0.35 M NaOH) was added to the incubation system at the time indicated by the arrow (time zero), in a 1:0.4 volume ratio with respect to the initial volume of incubation system. Due to its effect as phase separation inducer, 0.4 M Na_2CO_3 was also tested as inactivator only in the absence of PEG.

A_{420} vs. time data, starting at time zero, was submitted to a linear regression analysis. Table 1 shows the results obtained. Ordinates ("a") are A_{420} values at the enzyme inactivation time, and represent the maximum amount of ONPx produced during the initial incubation period. Slopes ("b") values close to zero reflect the efficacy of the inactivation procedure.

d) Effect of PEG_{6000} on ONPG non-enzymatic hydrolysis and β-Gal inactivation treatment induced by NaOH.

Table S1. Linear regression analysis of the time-dependent ONPG hydrolysis in the presence and absence of β-Gal using NaOH as inactivator.

[PEG_{6000}] (% w/v)	**Regression parameter**	**Sample treatment**	
		- β-Gal	**+ β-Gal**
0	b	0.005 ± 0.0004	0.001 ± 0.004
	a	-0.004 ± 0.001	0.35 ± 0.12 #
31	b	0.008 ± 0.005	0.001 ± 0.009
	a	0.18 ± 0.12 *	0.45 ± 0.20
45	b	0.018 ± 0.008	0.014 ± 0.009
	a	0.24 ± 0.17 *	0.39 ± 0.19

Samples (8 mM ONPG with or without β-Gal) were incubated at 37ºC during 10 min. After that, 0.4M NaOH was added and A_{420} was measured for 30 min. A linear equation was fitted to the experimental data. *Significantly different with respect to the control without PEG_{6000}; #significantly different with respect to the control without enzyme. b, slope; a, ordinate.

At 0% w/v PEG_{6000}, in the presence or in the absence of β-Gal, slopes were not statistically different from zero. This was a clear indication that, in non-crowded media, this procedure was effective as an enzyme inactivator. As expected, the ordinate values ("a") were zero in the absence of β-Gal and higher in samples containing β-Gal, due to the catalytic action of the enzyme on ONPG.

In the absence of the enzyme, the presence of PEG_{6000}, mainly at the highest concentration assayed (45% w/v), induced an increase in the slope "b" values with respect to those without PEG_{6000}, suggesting that a non-enzymatic PEG-dependent ONPG hydrolysis was occurring. In these samples, ordinates were also higher than those in the absence of PEG, strongly indicating that enzymatic ONPG hydrolysis was enhanced in crowded media, although effects on the spectroscopic behaviour of ONP could not be excluded.

e) Optimal time and enzyme concentration determination

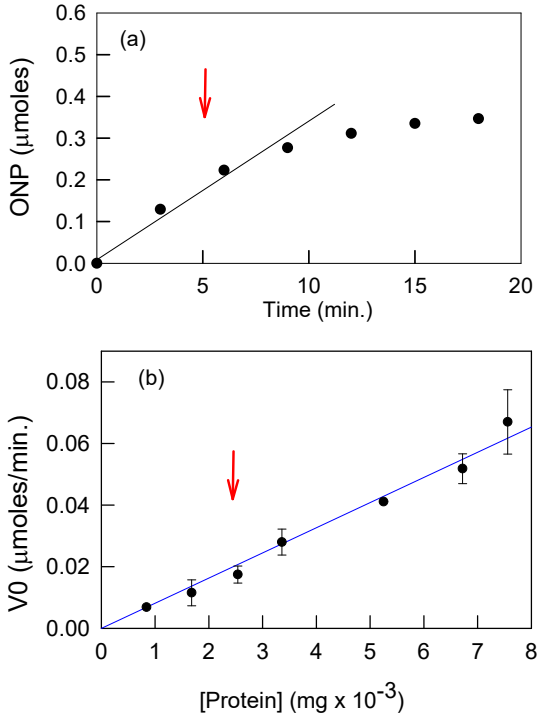

Fig.S3 Determination of optimal conditions of *K.lactis* β-Gal catalyzed ONPG hydrolysis. (a) Rate as a function of the incubation time in the presence of 2.54 x 10^{-3} mg/ml of enzyme and (b) Initial rate as a function of enzyme concentration measured at 5 min incubation time.

Eadie-Hofstee analysis of *Kluyveromyces lactis* catalyzed reaction rate as a function of [ONPG]. Effect of PEG

The shapes of these plots ratify the michaelian-type kinetics in the absence and in the presence of 15 and 25% w/v PEG (Fig.S3a) and the cooperative kinetics in the presence of 35% w/v PEG (Fig.3b). For comparisons of line shapes and types of kinetics involved, see the article of Hutzler and Tracy, (2001) [4].

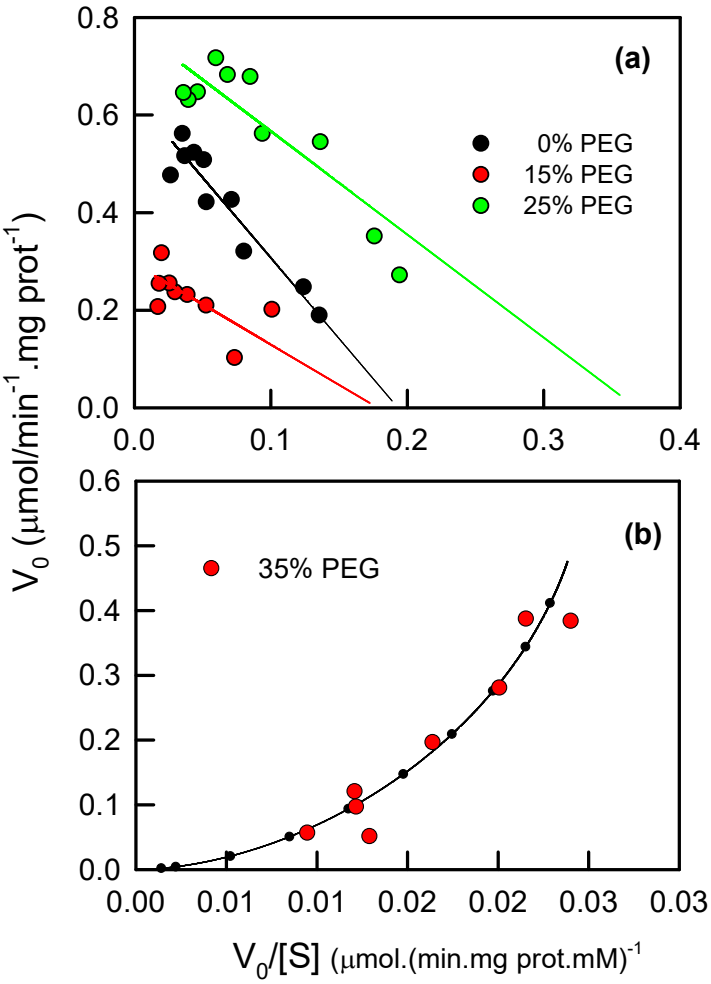

Fig.S4. Eadie-Hofstee plots. Data are the same shown in rate as a function of ONPG concentration depicted in Fig.1. a) Straight lines were fitted to data in the absence (●) and in the presence of 15% (●) and 25% PEG (●) as expected from Michaelis–Menten hyperbolic-type kinetics. Lines correspond to the linear regressions. ANOVA produced p=0.003, 0.001 y 0.067, respectively.

b) This Eadie-Hofstee plot has the shape expected from a sigmoidal kinetics. Red dots correspond to experimental points. Small black dots are calculated values.

Figure 2. Effect of PEG_{6000} on the thermal stability of β-Gal**.**

STATISTICAL ANALYSIS (ANOVA + TEST DE TUKEY) PARA "for raw data"
All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **temp within 0%PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 50.000	2.044	5	24.864	<0.001	Yes
20.000 vs. 45.000	1.998	5	24.302	<0.001	Yes
20.000 vs. 40.000	1.337	5	16.270	<0.001	Yes
20.000 vs. 30.000	0.406	5	4.934	0.007	Yes
30.000 vs. 50.000	1.638	5	19.929	<0.001	Yes
30.000 vs. 45.000	1.592	5	19.367	<0.001	Yes
30.000 vs. 40.000	0.932	5	11.336	<0.001	Yes
40.000 vs. 50.000	0.706	5	8.594	<0.001	Yes
40.000 vs. 45.000	0.660	5	8.032	<0.001	Yes
45.000 vs. 50.000	**0.0462**	**5**	**0.562**	**0.995**	**No**

Comparisons for factor: **temp within 15%PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 50.000	2.254	5	27.415	<0.001	Yes
20.000 vs. 45.000	1.828	5	22.242	<0.001	Yes
20.000 vs. 40.000	1.137	5	13.836	<0.001	Yes
20.000 vs. 30.000	**0.190**	**5**	**2.311**	**0.481**	**No**
30.000 vs. 50.000	2.064	5	25.104	<0.001	Yes
30.000 vs. 45.000	1.638	5	19.932	<0.001	Yes
30.000 vs. 40.000	0.947	5	11.525	<0.001	Yes
40.000 vs. 50.000	1.116	5	13.579	<0.001	Yes
40.000 vs. 45.000	0.691	5	8.406	<0.001	Yes
45.000 vs. 50.000	0.425	5	5.173	0.004	Yes

Comparisons for factor: **temp within 25%PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 50.000	2.541	5	30.911	<0.001	Yes
20.000 vs. 45.000	1.828	5	22.232	<0.001	Yes
20.000 vs. 40.000	0.573	5	6.969	<0.001	Yes
20.000 vs. 30.000	0.374	5	4.546	0.016	Yes
30.000 vs. 50.000	2.167	5	26.365	<0.001	Yes
30.000 vs. 45.000	1.454	5	17.686	<0.001	Yes
30.000 vs. 40.000	**0.199**	**5**	**2.423**	**0.432**	**No**
40.000 vs. 50.000	1.968	5	23.942	<0.001	Yes
40.000 vs. 45.000	1.255	5	15.263	<0.001	Yes
45.000 vs. 50.000	0.713	5	8.679	<0.001	Yes

Comparisons for factor: **temp within 35%PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 50.000	3.184	5	38.739	<0.001	Yes
20.000 vs. 45.000	2.193	5	26.679	<0.001	Yes
20.000 vs. 40.000	0.818	5	9.946	<0.001	Yes
20.000 vs. 30.000	0.693	5	8.435	<0.001	Yes
30.000 vs. 50.000	2.491	5	30.304	<0.001	Yes
30.000 vs. 45.000	1.500	5	18.243	<0.001	Yes
30.000 vs. 40.000	**0.124**	**5**	**1.511**	**0.822**	**No**
40.000 vs. 50.000	2.367	5	28.793	<0.001	Yes
40.000 vs. 45.000	1.375	5	16.732	<0.001	Yes
45.000 vs. 50.000	0.991	5	12.061	<0.001	Yes

Comparisons for factor: **PEG within 20°C**

Comparison	Diff of Means	p	q	P	P<0.050
35.000 vs. 0.000	1.152	4	14.012	<0.001	Yes
35.000 vs. 15.000	0.936	4	11.392	<0.001	Yes
35.000 vs. 25.000	0.635	4	7.729	<0.001	Yes
25.000 vs. 0.000	0.517	4	6.283	<0.001	Yes
25.000 vs. 15.000	**0.301**	**4**	**3.664**	**0.054**	**No**
15.000 vs. 0.000	**0.215**	**4**	**2.620**	**0.257**	**No**

Comparisons for factor: **PEG within 30°C**

Comparison	Diff of Means	p	q	P	P<0.050
35.000 vs. 0.000	0.864	4	10.511	<0.001	Yes
35.000 vs. 15.000	0.433	4	5.268	0.002	Yes
35.000 vs. 25.000	0.316	4	3.839	0.040	Yes
25.000 vs. 0.000	0.548	4	6.671	<0.001	Yes
25.000 vs. 15.000	**0.117**	**4**	**1.428**	**0.744**	**No**
15.000 vs. 0.000	0.431	4	5.243	0.002	Yes

Comparisons for factor: **PEG within 40°C**

Comparison	Diff of Means	p	q	P	P<0.050
35.000 vs. 0.000	1.672	4	20.336	<0.001	Yes
35.000 vs. 15.000	1.256	4	15.282	<0.001	Yes
35.000 vs. 25.000	0.391	4	4.751	0.007	Yes
25.000 vs. 0.000	1.281	4	15.584	<0.001	Yes
25.000 vs. 15.000	0.866	4	10.531	<0.001	Yes
15.000 vs. 0.000	0.415	4	5.053	0.003	Yes

Comparisons for factor: **PEG within 45°C**

Comparison	Diff of Means	p	q	P	P<0.050
35.000 vs. 0.000	0.956	4	11.635	<0.001	Yes
35.000 vs. 15.000	0.572	4	6.956	<0.001	Yes
35.000 vs. 25.000	**0.270**	**4**	**3.282**	**0.102**	**No**
25.000 vs. 0.000	0.687	4	8.353	<0.001	Yes
25.000 vs. 15.000	0.302	4	3.674	0.053	No
15.000 vs. 0.000	0.385	4	4.679	0.008	Yes

Comparisons for factor: **PEG within 50°C**

Comparison	Diff of Means	p	q	P	P<0.050
25.000 vs. 0.000	**0.0194**	**4**	**0.236**	**0.998**	**No**
25.000 vs. 15.000	0.0138	4	0.168	0.999	Do Not Test
25.000 vs. 35.000	0.00820	4	0.0998	1.000	Do Not Test
35.000 vs. 0.000	0.0112	4	0.136	1.000	Do Not Test
35.000 vs. 15.000	0.00560	4	0.0681	1.000	Do Not Test
15.000 vs. 0.000	0.00560	4	0.0681	1.000	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

STATISTICAL ANALYSIS (ANOVA + TEST DE TUKEY) "for percentages"
All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **PEG**

Comparison	Diff of Means	p	q	P	P<0.050
25.000 vs. 0.000	14.200	4	12.833	<0.001	Yes
25.000 vs. 15.000	5.800	4	5.242	0.004	Yes
25.000 vs. 35.000	**1.663**	**4**	**1.503**	**0.714**	**No**
35.000 vs. 0.000	12.537	4	11.330	<0.001	Yes
35.000 vs. 15.000	**4.137**	**4**	**3.738**	**0.055**	**No**
15.000 vs. 0.000	8.400	4	7.591	<0.001	Yes

Comparisons for factor: **Temp**

Comparison	Diff of Means	p	q	P	P<0.050
20.000 vs. 50.000	96.079	5	77.663	<0.001	Yes
20.000 vs. 45.000	77.000	5	62.241	<0.001	Yes
20.000 vs. 40.000	39.500	5	31.929	<0.001	Yes
20.000 vs. 30.000	15.500	5	12.529	<0.001	Yes
30.000 vs. 50.000	80.579	5	65.134	<0.001	Yes
30.000 vs. 45.000	61.500	5	49.712	<0.001	Yes
30.000 vs. 40.000	24.000	5	19.400	<0.001	Yes
40.000 vs. 50.000	56.579	5	45.734	<0.001	Yes
40.000 vs. 45.000	37.500	5	30.312	<0.001	Yes
45.000 vs. 50.000	19.079	5	15.422	<0.001	Yes

Comparisons for factor: **Temp within 0%PEG**

Comparison	Diff of Means	p	q	P	P<0.050
20.000 vs. 50.000	96.000	5	38.799	<0.001	Yes
20.000 vs. 45.000	94.000	5	37.991	<0.001	Yes
20.000 vs. 40.000	63.000	5	25.462	<0.001	Yes
20.000 vs. 30.000	**19.000**	**5**	**7.679**	**<0.001**	**Yes**
30.000 vs. 50.000	77.000	5	31.120	<0.001	Yes
30.000 vs. 45.000	75.000	5	30.312	<0.001	Yes
30.000 vs. 40.000	44.000	5	17.783	<0.001	Yes
40.000 vs. 50.000	33.000	5	13.337	<0.001	Yes
40.000 vs. 45.000	31.000	5	12.529	<0.001	Yes
45.000 vs. 50.000	2.000	5	0.808	0.979	No

Comparisons for factor: **Temp within 15%PEG**

Comparison	Diff of Means	p	q	P	P<0.050
20.000 vs. 50.000	96.000	5	38.799	<0.001	Yes
20.000 vs. 45.000	78.000	5	31.525	<0.001	Yes
20.000 vs. 40.000	48.000	5	19.400	<0.001	Yes
20.000 vs. 30.000	**8.000**	**5**	**3.233**	**0.171**	**No**
30.000 vs. 50.000	88.000	5	35.566	<0.001	Yes
30.000 vs. 45.000	70.000	5	28.291	<0.001	Yes
30.000 vs. 40.000	40.000	5	16.166	<0.001	Yes
40.000 vs. 50.000	48.000	5	19.400	<0.001	Yes
40.000 vs. 45.000	30.000	5	12.125	<0.001	Yes
45.000 vs. 50.000	18.000	5	7.275	<0.001	Yes

Comparisons for factor: **Temp within 25%PEG**

Comparison	Diff of Means	p	q	P	P<0.050
20.000 vs. 50.000	96.000	5	38.799	<0.001	Yes
20.000 vs. 45.000	69.000	5	27.887	<0.001	Yes

20.000 vs. 40.000	22.000	5	8.892	<0.001	Yes
20.000 vs. 30.000	**14.000**	**5**	**5.658**	**0.002**	**Yes**
30.000 vs. 50.000	82.000	5	33.141	<0.001	Yes
30.000 vs. 45.000	55.000	5	22.229	<0.001	Yes
30.000 vs. 40.000	**8.000**	**5**	**3.233**	**0.171**	**No**
40.000 vs. 50.000	74.000	5	29.908	<0.001	Yes
40.000 vs. 45.000	47.000	5	18.996	<0.001	Yes
45.000 vs. 50.000	27.000	5	10.912	<0.001	Yes

Comparisons for factor: **Temp within 35%PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
20.000 vs. 50.000	96.317	5	38.927	<0.001	Yes
20.000 vs. 45.000	67.000	5	27.079	<0.001	Yes
20.000 vs. 40.000	25.000	5	10.104	<0.001	Yes
20.000 vs. 30.000	**21.000**	**5**	**8.487**	**<0.001**	**Yes**
30.000 vs. 50.000	75.317	5	30.440	<0.001	Yes
30.000 vs. 45.000	46.000	5	18.591	<0.001	Yes
30.000 vs. 40.000	**4.000**	**5**	**1.617**	**0.783**	**No**
40.000 vs. 50.000	71.317	5	28.823	<0.001	Yes
40.000 vs. 45.000	42.000	5	16.975	<0.001	Yes
45.000 vs. 50.000	29.317	5	11.849	<0.001	Yes

Comparisons for factor: **PEG within 20°C (**correspond to the control; 100%**)**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
0.000 vs. 35.000	0.000	4	0.000	1.000	No
0.000 vs. 25.000	0.000	4	0.000	1.000	Do Not Test
0.000 vs. 15.000	0.000	4	0.000	1.000	Do Not Test
15.000 vs. 35.000	0.000	4	0.000	1.000	Do Not Test
15.000 vs. 25.000	0.000	4	0.000	1.000	Do Not Test
25.000 vs. 35.000	0.000	4	0.000	1.000	Do Not Test

Comparisons for factor: **PEG within 30°C**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
15.000 vs. 35.000	13.000	4	5.254	0.003	Yes
15.000 vs. 0.000	11.000	4	4.446	0.016	Yes
15.000 vs. 25.000	**6.000**	**4**	**2.425**	**0.330**	**No**
25.000 vs. 35.000	**7.000**	**4**	**2.829**	**0.205**	**No**
25.000 vs. 0.000	5.000	4	2.021	0.489	Do Not Test
0.000 vs. 35.000	2.000	4	0.808	0.940	Do Not Test

Comparisons for factor: **PEG within 40°C**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
25.000 vs. 0.000	41.000	4	16.571	<0.001	Yes
25.000 vs. 15.000	26.000	4	10.508	<0.001	Yes
25.000 vs. 35.000	**3.000**	**4**	**1.212**	**0.827**	**No**
35.000 vs. 0.000	38.000	4	15.358	<0.001	Yes
35.000 vs. 15.000	23.000	4	9.296	<0.001	Yes
15.000 vs. 0.000	15.000	4	6.062	<0.001	Yes

Comparisons for factor: **PEG within 45°C**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
35.000 vs. 0.000	27.000	4	10.912	<0.001	Yes
35.000 vs. 15.000	11.000	4	4.446	0.016	Yes
35.000 vs. 25.000	**2.000**	**4**	**0.808**	**0.940**	**No**

25.000 vs. 0.000	25.000	4	10.104	<0.001	Yes
25.000 vs. 15.000	**9.000**	**4**	**3.637**	**0.064**	**No**
15.000 vs. 0.000	16.000	4	6.467	<0.001	Yes

Comparisons for factor: **PEG within 50°C**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
0.000 vs. 35.000	**0.317**	**4**	**0.128**	**1.000**	**No**
0.000 vs. 25.000	4.441E-016	4	1.795E-016	1.000	Do Not Test
0.000 vs. 15.000	0.000	4	0.000	1.000	Do Not Test
15.000 vs. 35.000	0.317	4	0.128	1.000	Do Not Test
15.000 vs. 25.000	4.441E-016	4	1.795E-016	1.000	Do Not Test
25.000 vs. 35.000	0.317	4	0.128	1.000	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

Fig.3. Effect of PEG_{6000} on the thermal inactivation kinetics of β-Gal.

Raw data

Time (min)	Catalytic activity (μM.min^{-1}) (Preincubation at 30°C, PEG 0%)				
	Sample 1	**Sample 2**	**Sample 3**	**Media**	**s.e.m.**
0	3.65	3.94	3.98	3.85	0.102
10	3.36	3.93	3.93	3.74	0.189
20	3.06	3.49	4.01	3.52	0.275
30	3.18	3.82	3.85	3.62	0.217
40	3.23	3.51	3.39	3.38	0.081
50	2.92	3.26	3.23	3.14	0.110
60	2.89	3.55	3.17	3.20	0.192
70	2.91	2.98	2.92	2.94	0.229
80	3.10	3.30	3.29	3.23	0.630

Time (min)	Catalytic activity (μM.min^{-1}) (Preincubation at 30°C, PEG 25%)				
	Sample 1	**Sample 2**	**Sample 3**	**Media**	**s.e.m.**
0	5.94	4.90	5.64	5.50	0.309
10	5.80	6.17	6.28	6.08	0.145
20	7.12	5.37	5.19	5.90	0.616
30	5.47	5.75	5.43	5.55	0.100
40	5.82	5.89	6.24	5.99	0.128
50	5.75	5.69	5.45	5.63	0.928
60	4.96	5.71	5.80	5.49	0.267
70	6.52	5.11	5.54	5.73	0.417
80	5.42	5.38	5.93	5.58	0.176

Time (min)	Catalytic activity (μM.min^{-1}) (Preincubation at 45°C, PEG 0%)				
	Sample 1	**Sample 2**	**Sample 3**	**Media**	**s.e.m.**
0	2.06	2.04	2.07	2.06	0.00705
10	0.495	0.452	0.623	0.524	0.0512
20	0.47	0.408	0.434	0.437	0.0178
30	0.439	0.366	0.388	0.398	0.0217
40	0.452	0.546	0.366	0.454	0.0521
50	0.366	0.36	0.373	0.366	0.00371
60	0.408	0.387	0.37	0.388	0.0109
70	0.369	0.36	0.401	0.377	0.0124
80	0.388	0.359	0.375	0.374	0.00826

Time (min)	Catalytic activity (μM.min^{-1}) (Preincubation at 45°C, PEG 25%)				
	Sample 1	**Sample 2**	**Sample 3**	**Media**	**s.e.m.**
0	2.65	3.83	3.21	3.23	0.34
10	1.71	1.8	1.71	1.74	0.0275
20	1.36	1.33	0.878	1.19	0.156
30	1.06	1.03	1.11	1.07	0.0249
40	0.827	0.864	0.907	0.866	0.0232
50	0.758	0.884	0.723	0.789	0.049
60	0.801	0.636	0.639	0.692	0.0547
70	0.541	0.527	0.578	0.549	0.0152
80	0.44	0.476	0.448	0.455	0.0109

Two Way Analysis of Variance

Data source: Data 1 in Fig 3 paper K lactis.JNB
Balanced Design

Dependent Variable: 0%PEG 30°C

Equal Variance Test: Passed (P = 0.567)

Source of Variation	DF	SS	MS	F	P
Time	8	1.965	0.246	1.421	0.221
PEG %	1	72.153	72.153	417.484	<0.001
Time x PEG %	8	1.427	0.178	1.032	0.431
Residual	36	6.222	0.173		
Total	53	81.766	1.543		

The difference in the mean values among the different levels of Time is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in PEG %. There is not a statistically significant difference (P = 0.221).

The difference in the mean values among the different levels of PEG % is greater than would be expected by chance after allowing for effects of differences in Time. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Time does not depend on what level of PEG % is present. There is not a statistically significant interaction between Time and PEG %. (P = 0.431)

Power of performed test with alpha = 0.0500: for Time : 0.168
Power of performed test with alpha = 0.0500: for PEG % : 1.000
Power of performed test with alpha = 0.0500: for Time x PEG % : 0.0567

Least square means for Time :

Group	**Mean**
0.000	4.675
10.000	4.912
20.000	4.707
30.000	4.583
40.000	4.680
50.000	4.383
60.000	4.347
70.000	4.330
80.000	4.403

Std Err of LS Mean = 0.170

Least square means for PEG % :

Group	**Mean**
0.000	3.402
25.000	5.714

Std Err of LS Mean = 0.0800

Least square means for Time x PEG % :

Group	**Mean**
0.000 x 0.000	3.857
0.000 x 25.000	5.493
10.000 x 0.000	3.740
10.000 x 25.000	6.083
20.000 x 0.000	3.520
20.000 x 25.000	5.893
30.000 x 0.000	3.617
30.000 x 25.000	5.550
40.000 x 0.000	3.377
40.000 x 25.000	5.983
50.000 x 0.000	3.137
50.000 x 25.000	5.630
60.000 x 0.000	3.203
60.000 x 25.000	5.490
70.000 x 0.000	2.937
70.000 x 25.000	5.723
80.000 x 0.000	3.230
80.000 x 25.000	5.577

Std Err of LS Mean = 0.240

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Time**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
10.000 vs. 70.000	**0.582**	**9**	**3.427**	**0.303**	**No**
10.000 vs. 60.000	0.565	9	3.329	0.339	Do Not Test

10.000 vs. 50.000	0.528	9	3.113	0.426	Do Not Test
10.000 vs. 80.000	0.508	9	2.995	0.478	Do Not Test
10.000 vs. 30.000	0.328	9	1.935	0.902	Do Not Test
10.000 vs. 0.000	0.237	9	1.394	0.985	Do Not Test
10.000 vs. 40.000	0.232	9	1.365	0.987	Do Not Test
10.000 vs. 20.000	0.205	9	1.208	0.994	Do Not Test
20.000 vs. 70.000	0.377	9	2.219	0.815	Do Not Test
20.000 vs. 60.000	0.360	9	2.121	0.848	Do Not Test
20.000 vs. 50.000	0.323	9	1.905	0.910	Do Not Test
20.000 vs. 80.000	0.303	9	1.787	0.935	Do Not Test
20.000 vs. 30.000	0.123	9	0.727	1.000	Do Not Test
20.000 vs. 0.000	0.0317	9	0.187	1.000	Do Not Test
20.000 vs. 40.000	0.0267	9	0.157	1.000	Do Not Test
40.000 vs. 70.000	0.350	9	2.062	0.867	Do Not Test
40.000 vs. 60.000	0.333	9	1.964	0.895	Do Not Test
40.000 vs. 50.000	0.297	9	1.748	0.943	Do Not Test
40.000 vs. 80.000	0.277	9	1.630	0.961	Do Not Test
40.000 vs. 30.000	0.0967	9	0.570	1.000	Do Not Test
40.000 vs. 0.000	0.00500	9	0.0295	1.000	Do Not Test
0.000 vs. 70.000	0.345	9	2.033	0.876	Do Not Test
0.000 vs. 60.000	0.328	9	1.935	0.902	Do Not Test
0.000 vs. 50.000	0.292	9	1.719	0.948	Do Not Test
0.000 vs. 80.000	0.272	9	1.601	0.965	Do Not Test
0.000 vs. 30.000	0.0917	9	0.540	1.000	Do Not Test
30.000 vs. 70.000	0.253	9	1.493	0.977	Do Not Test
30.000 vs. 60.000	0.237	9	1.394	0.985	Do Not Test
30.000 vs. 50.000	0.200	9	1.178	0.995	Do Not Test
30.000 vs. 80.000	0.180	9	1.061	0.998	Do Not Test
80.000 vs. 70.000	0.0733	9	0.432	1.000	Do Not Test
80.000 vs. 60.000	0.0567	9	0.334	1.000	Do Not Test
80.000 vs. 50.000	0.0200	9	0.118	1.000	Do Not Test
50.000 vs. 70.000	0.0533	9	0.314	1.000	Do Not Test
50.000 vs. 60.000	0.0367	9	0.216	1.000	Do Not Test
60.000 vs. 70.000	0.0167	9	0.0982	1.000	Do Not Test

Comparisons for factor: **PEG %**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.050**
25.000 vs. 0.000	2.312	2	28.896	<0.001	Yes

Comparisons for factor: **PEG % within 0**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	1.637	2	6.819	<0.001	Yes

Comparisons for factor: **PEG % within 10**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.343	2	9.763	<0.001	Yes

Comparisons for factor: **PEG % within 20**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.373	2	9.888	<0.001	Yes

Comparisons for factor: **PEG % within 30**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	1.933	2	8.055	<0.001	Yes

Comparisons for factor: **PEG % within 40**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.607	2	10.860	<0.001	Yes

Comparisons for factor: **PEG % within 50**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.493	2	10.388	<0.001	Yes

Comparisons for factor: **PEG % within 60**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.287	2	9.527	<0.001	Yes

Comparisons for factor: **PEG % within 70**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.787	2	11.610	<0.001	Yes

Comparisons for factor: **PEG % within 80**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
25.000 vs. 0.000	2.347	2	9.777	<0.001	Yes

Comparisons for factor: **Time within 0% PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
0.000 vs. 70.000	**0.920**	**9**	**3.833**	**0.180**	**No**
0.000 vs. 50.000	0.720	9	3.000	0.476	Do Not Test
0.000 vs. 60.000	0.653	9	2.722	0.602	Do Not Test
0.000 vs. 80.000	0.627	9	2.611	0.653	Do Not Test
0.000 vs. 40.000	0.480	9	2.000	0.885	Do Not Test
0.000 vs. 20.000	0.337	9	1.403	0.984	Do Not Test
0.000 vs. 30.000	0.240	9	1.000	0.998	Do Not Test
0.000 vs. 10.000	0.117	9	0.486	1.000	Do Not Test
10.000 vs. 70.000	0.803	9	3.347	0.332	Do Not Test
10.000 vs. 50.000	0.603	9	2.514	0.696	Do Not Test
10.000 vs. 60.000	0.537	9	2.236	0.808	Do Not Test
10.000 vs. 80.000	0.510	9	2.125	0.847	Do Not Test
10.000 vs. 40.000	0.363	9	1.514	0.975	Do Not Test
10.000 vs. 20.000	0.220	9	0.917	0.999	Do Not Test
10.000 vs. 30.000	0.123	9	0.514	1.000	Do Not Test
30.000 vs. 70.000	0.680	9	2.833	0.551	Do Not Test
30.000 vs. 50.000	0.480	9	2.000	0.885	Do Not Test
30.000 vs. 60.000	0.413	9	1.722	0.947	Do Not Test
30.000 vs. 80.000	0.387	9	1.611	0.964	Do Not Test
30.000 vs. 40.000	0.240	9	1.000	0.998	Do Not Test
30.000 vs. 20.000	0.0967	9	0.403	1.000	Do Not Test
20.000 vs. 70.000	0.583	9	2.430	0.732	Do Not Test
20.000 vs. 50.000	0.383	9	1.597	0.966	Do Not Test
20.000 vs. 60.000	0.317	9	1.319	0.989	Do Not Test
20.000 vs. 80.000	0.290	9	1.208	0.994	Do Not Test
20.000 vs. 40.000	0.143	9	0.597	1.000	Do Not Test
40.000 vs. 70.000	0.440	9	1.833	0.926	Do Not Test
40.000 vs. 50.000	0.240	9	1.000	0.998	Do Not Test
40.000 vs. 60.000	0.173	9	0.722	1.000	Do Not Test
40.000 vs. 80.000	0.147	9	0.611	1.000	Do Not Test

80.000 vs. 70.000	0.293	9	1.222	0.994	Do Not Test
80.000 vs. 50.000	0.0933	9	0.389	1.000	Do Not Test
80.000 vs. 60.000	0.0267	9	0.111	1.000	Do Not Test
60.000 vs. 70.000	0.267	9	1.111	0.997	Do Not Test
60.000 vs. 50.000	0.0667	9	0.278	1.000	Do Not Test
50.000 vs. 70.000	0.200	9	0.833	1.000	Do Not Test

Comparisons for factor: **Time within 25% PEG**

Comparison	**Diff of Means**	**p**	**q**	**P**	**P<0.05**
10.000 vs. 60.000	**0.593**	**9**	**2.472**	**0.714**	**No**
10.000 vs. 0.000	0.590	9	2.458	0.720	Do Not Test
10.000 vs. 30.000	0.533	9	2.222	0.814	Do Not Test
10.000 vs. 80.000	0.507	9	2.111	0.852	Do Not Test
10.000 vs. 50.000	0.453	9	1.889	0.914	Do Not Test
10.000 vs. 70.000	0.360	9	1.500	0.976	Do Not Test
10.000 vs. 20.000	0.190	9	0.792	1.000	Do Not Test
10.000 vs. 40.000	0.1000	9	0.417	1.000	Do Not Test
40.000 vs. 60.000	0.493	9	2.055	0.869	Do Not Test
40.000 vs. 0.000	0.490	9	2.042	0.873	Do Not Test
40.000 vs. 30.000	0.433	9	1.805	0.932	Do Not Test
40.000 vs. 80.000	0.407	9	1.694	0.952	Do Not Test
40.000 vs. 50.000	0.353	9	1.472	0.979	Do Not Test
40.000 vs. 70.000	0.260	9	1.083	0.997	Do Not Test
40.000 vs. 20.000	0.0900	9	0.375	1.000	Do Not Test
20.000 vs. 60.000	0.403	9	1.680	0.954	Do Not Test
20.000 vs. 0.000	0.400	9	1.667	0.956	Do Not Test
20.000 vs. 30.000	0.343	9	1.430	0.982	Do Not Test
20.000 vs. 80.000	0.317	9	1.319	0.989	Do Not Test
20.000 vs. 50.000	0.263	9	1.097	0.997	Do Not Test
20.000 vs. 70.000	0.170	9	0.708	1.000	Do Not Test
70.000 vs. 60.000	0.233	9	0.972	0.999	Do Not Test
70.000 vs. 0.000	0.230	9	0.958	0.999	Do Not Test
70.000 vs. 30.000	0.173	9	0.722	1.000	Do Not Test
70.000 vs. 80.000	0.147	9	0.611	1.000	Do Not Test
70.000 vs. 50.000	0.0933	9	0.389	1.000	Do Not Test
50.000 vs. 60.000	0.140	9	0.583	1.000	Do Not Test
50.000 vs. 0.000	0.137	9	0.569	1.000	Do Not Test
50.000 vs. 30.000	0.0800	9	0.333	1.000	Do Not Test
50.000 vs. 80.000	0.0533	9	0.222	1.000	Do Not Test
80.000 vs. 60.000	0.0867	9	0.361	1.000	Do Not Test
80.000 vs. 0.000	0.0833	9	0.347	1.000	Do Not Test
80.000 vs. 30.000	0.0267	9	0.111	1.000	Do Not Test
30.000 vs. 60.000	0.0600	9	0.250	1.000	Do Not Test
30.000 vs. 0.000	0.0567	9	0.236	1.000	Do Not Test
0.000 vs. 60.000	0.00333	9	0.0139	1.000	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison. For example, if you had four means sorted in order, and found no difference between means 4 vs. 2, then you would not test 4 vs. 3 and 3 vs. 2, but still test 4 vs. 1 and 3 vs. 1 (4 vs. 3 and 3 vs. 2 are enclosed by 4 vs. 2: 4 3 2 1). Note that not testing the enclosed means is a procedural rule, and a result of Do Not Test should be treated as if there is no significant difference between the means, even though one may appear to exist.

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