Cellular viscosity in prokaryotes and thermal stability of low-molecular weight biomolecules

A. Cuecas[1], J. Cruces[1], J. F. Galisteo-López[2], X. Peng[3], J.M. Gonzalez[1]*

[1] Institute of Natural Resources and Agrobiology, Spanish National Research Council, IRNAS-CSIC, Avda. Reina Mercedes 10, 41012 - Sevilla, Spain

[2] Multifunctional Optical Materials Group, Institute of Materials Science of Seville, Spanish National Research Council (CSIC), Americo Vespucio 49, 41092 – Sevilla, Spain

[3] State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Hi-tech Zone, Dalian 116024, P.R. China

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*To whom correspondence should be addressed:
J.M. Gonzalez
IRNAS-CSIC
Avda. Reina Mercedes 10
41012 – Sevilla, Spain
Tel. +34 95 462 4711; Fax +34 95 462 4002

Abstract

Some low-molecular weight biomolecules, i.e. NAD(P)H, are unstable at high temperatures. The use of these biomolecules by thermophilic microorganisms has been scarcely analyzed. Herein, NADH stability has been studied at different temperatures and viscosities. NADH decay increased at increasing temperatures. At increasing viscosities NADH decay rates decreased. Thus, maintaining relatively high cellular viscosity in cells could result in increased stability of low-molecular weight biomolecules (i.e., NADH) at high temperatures unlike previously deduced from studies in diluted water solutions. Cellular viscosity was determined using a fluorescent molecular rotor in various prokaryotes covering the range from 10ºC to 100ºC. Some mesophiles showed the capability of changing cellular viscosity depending on growth temperature. Thermophiles and extreme thermophiles presented a relatively high cellular viscosity suggesting this strategy as a reasonable mechanism to thrive under these high temperatures. Results substantiate the capability of thermophiles and extreme thermophiles (growth range 50ºC-80ºC) to stabilize and use generally considered unstable, universal low-molecular weight biomolecules. Besides, this study represents a first report on cellular viscosity measurements in prokaryotes and it shows the dependency of prokaryotic cellular viscosity on species and growth temperature.

Introduction

The upper temperature limit at which microorganisms can grow has been widely discussed (1,2). Currently, there is evidence of cell proliferation at 122ºC by *Methanopyrus kandleri* strain 116 isolated from deep-sea vents (3). It is known that these microorganisms use essentially the same biomolecules and similar metabolic machinery than all other living beings (1,2). However, some of the biomolecules required for cell functioning and growth (i.e., nicotinamide dinucleotides, adenosine triphosphate and phosphoribosyl phosphate among others) are unstable at high temperature (4). Therefore, there must be mechanisms to provide stability to essential biomolecules for life to be feasible in hot environments (2,5).

Numerous studies have attempted to explain the stability at high temperatures of essential macrobiomolecules, such as DNA and proteins (6,7). The stability of certain proteins under high temperature conditions is explained both by intrinsic mechanisms (modifications in sequence and structure) and by extrinsic mechanisms (interactions depending on solution composition) (7-9). Research on protein thermostability at high temperatures has explained relevant aspects of their functionality under extreme conditions (7).

However, living cells also depend on other small, low-molecular weight biomolecules, like redox and energy cofactors, required for cell functioning. A number of these molecules show high instability at high temperatures (2,10) suggesting that there must be some mechanisms for thermophiles to overcome this limitation under high temperature (4). For instance, although some factors like pH and certain ions have influence on NADH stability (11), there are no known mechanisms that explain NADH stability under high temperature (2,4).

Understanding the thermostability of small biomolecules is crucial to explain how thermophilic microorganisms are able to thrive under elevated temperatures (1).

It has been well described that cells accumulate some specific compatible solutes under osmotic stress (12,13). Several studies have shown that some compatible solutes are also accumulated in the cytoplasm of hyperthermophiles under conditions of heat stress (14-16). The stabilization of some enzymes at high temperatures has also been shown *in vitro* in the presence of some compatible solutes like betaine, choline, 1-glyceryl-1-myo-inosityl phosphate, glycine, ectoine or trehalose among others (14,16-18). Despite these results, the mechanisms conferring thermal stability by these solutes remain in most cases to be deciphered.

The cytoplasm of cells can be considered as a highly concentrated solution of multitude of interacting molecules, as the model of "molecular crowding" suggests (19-21). Consequently, its consistency is expected to be more like a viscous gel than an aqueous solution (20,22), which can influence several cellular properties (23). For instance, viscosity has been shown to play an important role in maintaining cell structure and function (22,24,25). Viscosity decreases with temperature (26). A viscous solution greatly reduces the physical impacts among biomolecules and so, it should result in higher stability (27,28). High temperature will induce a decrease in cell viscosity (26) and so, molecular and cell stability could be jeopardized.

Intracellular viscosity strongly influences diffusion and interaction between biomolecules (20,22,28). However, estimating intracellular viscosity is a difficult task. Traditional methods (i.e., mechanical viscosimeters) cannot be used to determine viscosity inside living cells. To

solve this problem, fluorescent ratiometric methods have been introduced (29). More recently, the use of fluorescent molecular rotors has been proposed to accurately determine intracellular viscosity (30). These molecular rotors typically comprise a conjugated domain that can freely rotate in low-viscosity solutions but viscous environments limit that action. This rotation is reflected in fluorescence intensity. Peng et al. (30) reported a new generation fluorescent molecular rotor, RY3, which provides dual emission peaks (456 nm, blue, and 650 nm, red) showing minimum fluorescence in non-viscous environments. Red fluorescence increases with viscosity while blue fluorescence remains insensitive, providing the basis for ratiometric measurements using a small molecule fluorescence sensor with cell-membrane permeability to investigate on intracellular viscosity (30). Results of cellular viscosity have been carried out only on eukaryotic cells. Viscosity reported for the cytoplasm of cells from tissue cultures presented values only slightly above those expected for water or dilute solutions (29).

The aim of this study is to infer whether viscosity could represent a potential factor to partially maintain the thermostability of small biomolecules (i.e., NADH) at increasing temperatures. NADH decay rates as a function of temperature and viscosity were evaluated. Besides, cellular viscosity was determined for prokaryotes over a broad temperature range to evaluate the relevance of the proposed strategy. Understanding how labile, low-molecular weight biomolecules can be maintained stable at high temperatures contributes to explain how thermophiles are able to thrive under extreme temperatures using mainly the same biomolecules than mesophiles.

Materials and Methods

NADH (nicotinamide adenine dinucleotide) was the selected low-molecular weight biomolecule due to the simplicity of use and lower cost (31). Differences in stability of NADH were tested as a function of temperature and viscosity. Dynamic viscosity (herein viscosity) (mPa·s) was considered in this study. Viscosity is dependent on temperature (26). Increased viscosity was obtained by supplementing assay solutions with either ethylene glycol or ectoine. Many other solutions were tested but most showed interference with the NADH assay. Viscosity was determined using a VisioLab AMVn micro-viscosimeter (Anton-Paar Inc., Graz, Austria) following the manufacturer's recommendations.

A first type of experiments aimed to determine the effect of viscosity on the stability of NADH over a range of temperatures (20ºC to 90ºC). These experiments were carried out in solution of phosphate buffer (pH 7.7) at concentrations of 0.05M and 0.1M for ethylene glycol (50% vol/vol) or ectoine (0.45 mg ml^{-1}) solutions, respectively. These buffering conditions resulted in a maximum stability for NADH (11). A buffered-water control lacking these compounds (ethylene glycol and ectoine) was also carried out. NADH was added to these assay solutions at 1 mM final concentration. Independent triplicate NADH solutions were assayed. Incubation was carried out over the temperature range from 20ºC to 90ºC. Decomposition of NADH was followed over time collecting aliquots at specific time periods up to a maximum of 30-50 h or until NADH decreased down to undetectable levels. NADH was quantified spectrophotometrically at 340 nm (32) using a Nanodrop 2000c (Thermo Scientific Inc., Wilmington, U.S.A.). As previously studied (33), the biologically oxidized form (NAD^+) is not the main intermediate. The major products of NADH thermal degradation resulted from the hydrolysis and oxidative ring opening of the reduced nicotinamide (33). Preliminary results showed that NADH decay followed first-order kinetics and the NADH decay rate was estimated by linear regression (34) plotting the natural logarithm of NADH

concentration *versus* time. NADH decay rates are expressed as absolute values so that the highest absolute value corresponds to the fastest decay and lowest stability. ANOVA and comparison of linear regression coefficients were used to evaluate the significance of differences between estimates following Sokal and Rohlf (34).

A second type of experiments aimed to determine the direct effect of viscosity on NADH decay at a constant temperature. In these experiments, a series of solutions with different viscosities were prepared. These viscosities were achieved by using the following concentrations of ethylene glycol: 0% (lacking ethylene glycol), 10%, 20%, 30%, 40%, 50%, and 60% vol/vol. Each one of these solutions was assayed at 20ºC, 50ºC, 70ºC and 90ºC. Assay conditions and estimates were carried out as described above. Unlike the first type of experiments (previous paragraph), this second type of experiments allowed to monitor the effect of viscosity at a given temperature.

Intracellular viscosity was estimated using a fluorescent molecular rotor, RY3 (30). RY3 was synthesized as previously described (30). For these experiments different prokaryotic strains were used, the bacteria: *Escherichia coli* K12 (CECT 433) (20ºC, 30ºC, 40ºC), *Pseudomonas aeroginosa* PAO1 (CECT 4122)(20ºC, 40ºC), *Lactococcus lactis lactis* (CECT 4433)(10ºC, 15ºC, 20ºC, 30ºC, 40ºC), *Geobacillus thermoglucosidasius* (CECT 4038)(50ºC, 60ºC, 70ºC), and *Fervidobacterium* FC2004 (JCM 18757)(60ºC, 70ºC, 80ºC), and the archaeon *Pyrococcus furiosus* (DSM 3638)(70ºC, 80ºC, 90ºC, 99ºC). The growth temperatures for those species covered the temperature range from 10ºC to 100ºC. Each strain was grown in the recommended culture medium under the required temperature condition. Cultures were harvested by centrifugation, washed with saline solution (0.9% NaCl) or a salt solution (recommended culture medium lacking organic components) for marine species. Cell

suspensions were adjusted to an OD 1. RY3 was added to cell suspensions at 5 µM final concentration (30) and incubated at the temperature of growth for 1 h. Ratiometric fluorescence measurements were carried out as described by Peng et al. (30) at the excitation (400 nm) and emission (456 nm and 650 nm) peaks. The intensity (I) of blue emission at 456 nm was insensitive to changes in viscosity and the red fluorescence intensity (650 nm) increased with viscosity. Ratiometric changes with viscosity followed a linear relationship between $\log(I_{650}/I_{456})$ and log(viscosity) which fitted the Föster-Hoffmann model (35). This calibration was achieved with different solutions of ethylene glycol (from 10% to 60%). Viscosity *versus* growth temperature was analyzed by non-linear curve fitting using the Log-Normal (3 parameters) function available in SigmaPlot software (Systat Software Inc., London, UK). To estimate the potential interference of RY3 dye and biomolecules in the tested cells, fluorescence lifetime measurements were performed using a Time Correlated Single Photon Counting (TCSPC) module (Fluorohub from Horiba). Samples were optically excited with 900 ps long pulses from a tunable laser source (Fianium SC400) operating at λ=633nm and fluorescence was collected at λ=715nm. The fluorescence decay curves were best fitted ($1.1 < \chi^2 < 1.3$) to a bi-exponential function:

$$I(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

and an intensity weighted average decay time was extracted from:

$$\tau_{avg} = \frac{\sum A_i \tau_i^2}{\sum A_i \tau_i}$$

Results

The persistence of NADH in solutions with different viscosities was analyzed within a wide temperature range (20-90ºC). Figure 1 shows the relationships between temperature and

viscosity for the solutions used in this study. Water viscosity decreases 3-fold from 20ºC to 80ºC. Solutions of ethylene glycol and ectoine were assayed to obtain higher viscosities than water.

Effects of temperature and viscosity on NADH decay. Stability of NADH in solutions at different viscosities and temperatures was assayed over time in ethylene glycol (50%, final concentration) or ectoine (0.45 mg ml^{-1}). A similar pattern was observed in both solutions (Figure 2). At the lowest temperature tested (20ºC) NADH remained stable over the time period assayed in this study both in buffered-water and the ethylene glycol solution (Figure 2A). Increasing temperatures resulted in a sharp increase of NADH decay. At temperatures of 50ºC and above (up to 90ºC) NADH decay rates at high viscosity (i.e., ethylene glycol solution) were significantly lower (as absolute values) than in control solutions at low viscosity (i.e., buffered-water) at the same temperature ($P<0.001$) (Figure 2A).

NADH decay in ectoine containing solutions (i.e., high viscosity) showed a lower decrease rate ($P<0.001$) than in the ectoine-lacking solutions (i.e., low viscosity control solution) over a range of temperatures (40ºC-80ºC) (Figure 2B). At 20ºC, NADH remained approximately constant over the length of the experiment. Increasing temperatures resulted in increasing NADH decay rates (Figure 2B).

Stabilization of NADH by viscosity. These experiments to evaluate the stabilization of NADH by viscosity were performed at four different temperatures (20ºC, 50ºC, 70ºC, and 90ºC). Results are shown in Figure 3. At 90ºC, increasing viscosities resulted in decreasing NADH decays for a range of viscosities from 0.4 to 1.1 mPa·s. At 70ºC and 50ºC, an increase of viscosity also resulted in decreases of the rates of NADH decay with viscosities 0.5-1.5

mPa·s and 0.6-2.4 mPa·s, respectively. At a given temperature (at and above 50ºC), increasing viscosity resulted in higher NADH stability (Figure 3). At 20ºC, NADH showed practically no decay during these experiments at a range of viscosities from about 1 mPa·s to over 5 mPa·s. The differences of NADH decay rates between the lowest and highest viscosities were comparatively decreasing as temperature decreased (Figure 4). At low viscosity (i.e. about 0.5 mPa·s) the effect of temperature was dramatic, leading to increasing NADH decay rates with temperature up to 16 h^{-1} at 90ºC. When comparing these effects at high viscosities (i.e. around 1.5 mPa·s), NADH remained much more stable over the full temperature range tested (20-90ºC) showing null decay (at 20ºC) and decays below 3 h^{-1} (at higher temperatures).

Curves of NADH decay rate versus viscosity (Figure 4) showed a reduction of NADH decay at increasing viscosity. The curves at the tested temperatures (Figure 4) pointed towards minimum decays at increasingly high viscosity values and drastic increases of NADH decay rates at low viscosities and high temperatures.

Cellular viscosity. Different prokaryotic strains showed different cellular viscosity. Figure 5 presents the viscosity estimates as a function of growth temperature for different prokaryotes. *Escherichia* and *Pseudomonas* growing in the temperature range from 20ºC to 40ºC presented very similar values and low cellular viscosity around the viscosity of water. The *Lactococcus* strain resulted in a large variation of viscosity at different temperatures. *Lactococcus* cells grown at the lowest studied temperature (10ºC) showed much lower cellular viscosity than these cells growing at 15ºC-20ºC and above. *Lactococcus* cells presented a peak of maximum viscosity at 15ºC-20ºC decreasing at higher and lower temperatures. The decrease of cellular viscosity in *Lactococcous* cells at 10ºC might be explained by a physiological adaptation to the limit imposed by exponentially increased viscosity at lowering temperatures. *Geobacillus*

cells are thermophiles growing from 50ºC to 70ºC showed lower cellular viscosity than *Lactococcus* cells but higher than *Fervidobacterium* cells grown from 60ºC to 80ºC. *Pyrococcus* cells grown from 70ºC to 99ºC presented the lowest viscosity values determined during this study and around the range of viscosity expected for water in its growth temperature range. For comparison purposes, Figure 5 presents the lines corresponding to the relationships viscosity *vs*. temperature for water and a solution of 60% ethylene glycol which are indicative of water and high viscosity conditions, respectively, used during the NADH decay experiments described above. The predicted line resulting from non-linear curve-fitting analysis including the data points from *Lactococcus*, *Geobacillus* and *Fervidobacterium* ($P<0.0001$; $r = 0.95$; $n = 11$) is also plotted in Figure 5. Estimated cellular viscosity of *Escherichia*, *Pseudomonas* and *Pyrococcus* was in the range of viscosity expected for water at their growth temperatures. *Lactococcus*, *Geobacillus* and *Fervidobacterium* presented values of cellular viscosity much higher than water at their growth temperatures but in the range of viscosity corresponding to a 60% ethylene glycol solution.

Fluorescence decay curves (Figure 6) showed no interference between RY3 dye and biomolecules in cells (where a faster decay rate would be expected as a consequence of potential fluorescence quenching). These curves presented similar patterns under the tested conditions (with and without cells, and for different cell types, *Pyrococcus* and *Lactococcus*). Estimates of τ_{avg} values were lower at high temperature (Figure 6). The τ_{avg} value for *Pyrococcus* cells was higher than the control lacking cells. The high τ_{avg} value obtained for *Lactococcus* confirmed the much higher cellular viscosity than can be reached in this bacterium.

Discussion

A number of biomolecules required for cell functioning are known to show *in vitro* instability at high temperatures (2,4). All living cells including thermophiles, use basically the same types of biomolecules. Several studies have attempted to clarify this issue focusing mainly on protein structure (7-9). Besides proteins, cell functioning requires numerous low-molecular weight biomolecules. For instance, NADH has been reported to be unstable at elevated temperatures (4). NADH is essential for cells by participating in numerous central metabolic processes involving electron transfers. This study shows evidence of the influence of cellular viscosity on the stability of low-molecular weight biomolecules at elevated temperatures and centers on the model provided by NADH.

The reported thermal instability of NADH (2,4,11) has been confirmed in this study showing increased decay at increasing temperatures. At temperatures of 50ºC and above, NADH showed clear decline over time which is in agreement to previous observations (33). Increasing viscosity resulted in increasing stability of NADH at various temperatures. Higher-than-water viscous solutions were obtained by different concentrations of ethylene glycol or supplementing with ectoine. For example, ectoine has been reported in thermophilic cells as a compatible solute which could be either synthesized by the cells or taken up from the medium contributing to cell survival at increasing temperatures (36,37). Previous studies have proposed other strategies to overcome the instability of biomolecules at high temperature but the actual mechanisms remain to be deciphered (1,4). High viscosity has been reported to be associated to cells adapted to heat stress in fungal spores (24) representing a preliminary example that maintaining viscosity could facilitate long-term preservation and the thermal stability of biomolecules. As a consequence, one could propose that the accumulation in thermophilic cells of a variety of solutes could be a possible mechanism to maintain

cytoplasm viscosity and compensate, at least partially, for the expected decrease of viscosity in the cell interior at increasing temperatures.

Viscosity greatly decreases with temperature and life at high temperatures is expected to be adapted to this phenomenon. Results have showed that viscous solutions contributed to significantly enhance the stability of NADH at high temperatures. Thus, maintaining viscosity in the cytoplasm of thermophiles could represent a potential mechanism to increase the stability of low-molecular weight biomolecules under elevated temperatures.

In order to evaluate the relevance of cellular viscosity to stabilize low-molecular weight biomolecules at high temperature, cellular viscosity was estimated for different prokaryotes using a novel fluorescent molecular rotor. This study is the first report comparing cellular viscosity among prokaryotes from a wide temperature range (from 10ºC to 100ºC). These results indicated that cellular viscosity (i) is species dependent and (ii) it can depend on growth temperature.

In this study we have observed two basic strategies of cellular viscosity in prokaryotes: A, those species that maintain relatively high cellular viscosity (around the values of a 60% ethylene glycol solution); B, those that present relatively low cellular viscosity (in the viscosity range of water and diluted solutions). Prokaryotes following the first strategy (A) should be potentially able to overcome, at least partially, the instability of low-molecular weight biomolecules at high temperatures by maintaining relatively high viscosities. This strategy fits with the observation that thermophilic cells are able to accumulate organic solutes (14-16) which could regulate cellular viscosity. This group includes thermophiles and extreme thermophiles and taxonomic phyla, such as Firmicutes and Thermotogae among the tested

species, with growing temperatures in the range 50ºC to 80ºC. An interesting example from this study is *Lactococcus* which presented a clear response of its cellular viscosity as a function of growth temperature. *Lactococcus* maximum cellular viscosity was observed for cells growing at 15ºC-20ºC. *Lactococcus* showed the ability to drastically modify its cellular viscosity as a response to growth at low (10ºC) and moderately high (30ºC-40ºC) temperature conditions. This is a first report showing the ability of bacterial cells to regulate its cellular viscosity depending on growing temperature.

The Gammaproteobacteria examined in this study correspond to typical mesophilic, gram-negative, bacteria and followed the second strategy (B). These species presented a cellular viscosity at the level of water within the 20ºC to 40ºC temperature range. These levels of viscosity present no compromise for biomolecule instability at that temperature range. However, a hyperthermophilic archaeon, *Pyrococcus*, also showed cellular viscosities around the expected values for water in spite of growing at the highest temperatures herein tested. Fluorescence decay curves indicated a lack of interference between RY3 dye and biomolecules in cells over a wide temperature range. The τ_{avg} obtained for *Pyrococcus* cells at 80ºC showed that these cells present cellular viscosity slightly above the water value which can only partially contribute to stabilizing small biomolecules. The decrease of viscosity with temperature suggests the existence of distinct or complementary physiological mechanisms to circumvent the thermal instability of key low-molecular weight biomolecules in hyperthermophiles (i.e., above 80ºC). Some potential mechanisms have been proposed including bearable rapid turnover rates, metabolite channelling and other strategies of local stabilization (4).

Conclusions

Cellular viscosity in prokaryotes is species dependent and it can vary as a result of growth temperature within a species. Increasing cellular viscosity enhances NADH stability at high temperature. Cellular viscosity observed for thermophiles and extreme thermophiles (growing optimally between 50ºC and 80ºC) corresponds to values of viscosity able to provide effective stability for NADH and likely other low-molecular weight biomolecules. Some mesophiles, such as *Lactococcus*, are capable to modify cellular viscosity depending on growing temperature likely as an adaptative mechanism to low/high temperature stress. On the other hand, some other cells (Gammaproteobacteria, *Pyrococcus*) present relatively low cellular viscosity at the level of water values. This study significantly contributes to explain the stability of low-molecular weight biomolecules in thermophiles and extreme thermophiles and to determine the cellular viscosity in prokaryotes and its variability.

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Author Contributions

AC performed most experiments with the help of JC. XP contributed with RY3 and participated in the cellular viscosity measurements. JMG designed the experiments and wrote

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most of the manuscript. J.F.G.-L performed and analyzed the fluorescence decay curves. All authors contributed to improve the final drafts of the manuscript.

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compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic bacterium *Chromohalobacter salexigens*. J. Bacteriol. 188:3774-3784.

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FIGURE LEGENDS

Figure 1. Effect of temperature on viscosity of the different solutions used in this study: buffered-water (dotted line with black diamonds), ectoine (0.45 mg ml^{-1})(dashed line with open squares), and ethylene glycol at 10% (open triangles), 20% (grey triangles), 30% (black triangles), 40% (open circles), 50% (grey circles), 60% (black circles). Error bars are shown except when covered by filled symbols.

Figure 2. NADH persistence in buffered-water (dotted lines with open symbols) and ethylene glycol (A) or ectoine (B) containing solutions with high viscosities (filled symbols) at different temperatures: 20ºC (black circles), 40ºC (grey squares), 50ºC (black squares), 60ºC (grey triangles), 70ºC (black triangles), 80ºC (grey diamonds) and 90ºC (black diamonds). Error bars are shown except when covered by filled symbols.

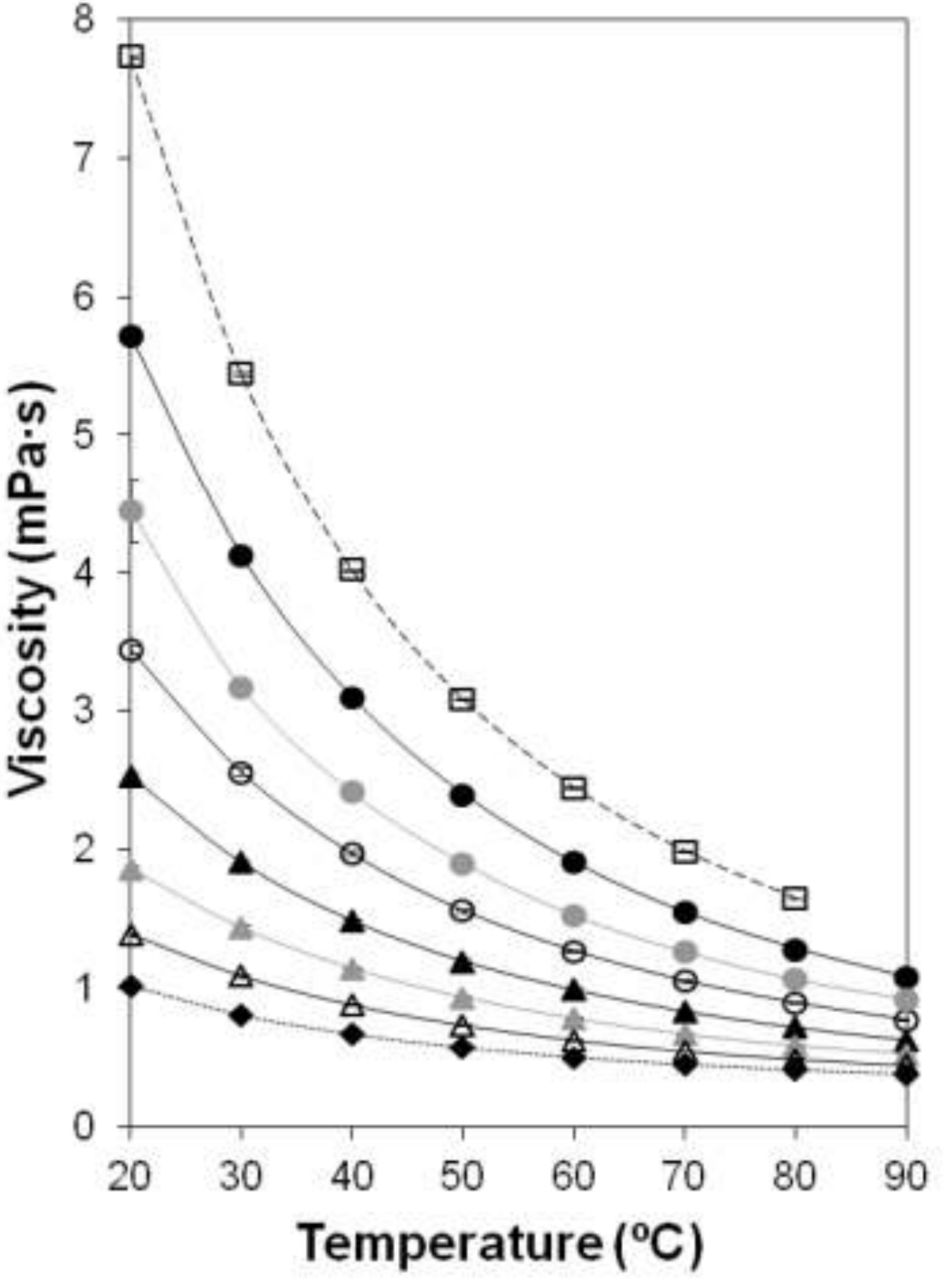
Figure 3. NADH persistence at different viscosities. The tested range of viscosities was prepared by supplementing the solutions with ethylene glycol at 0% (unsupplemented, ethylene glycol lacking solution; open symbols), 10% (grey squares), 20% (grey triangles), 30% (grey diamonds), 40% (black squares), 50% (black triangles), and 60% (black diamonds) final concentrations. The experiments were performed at 20ºC, 50ºC, 70ºC and 90ºC. Error bars are shown except when covered by filled symbols.

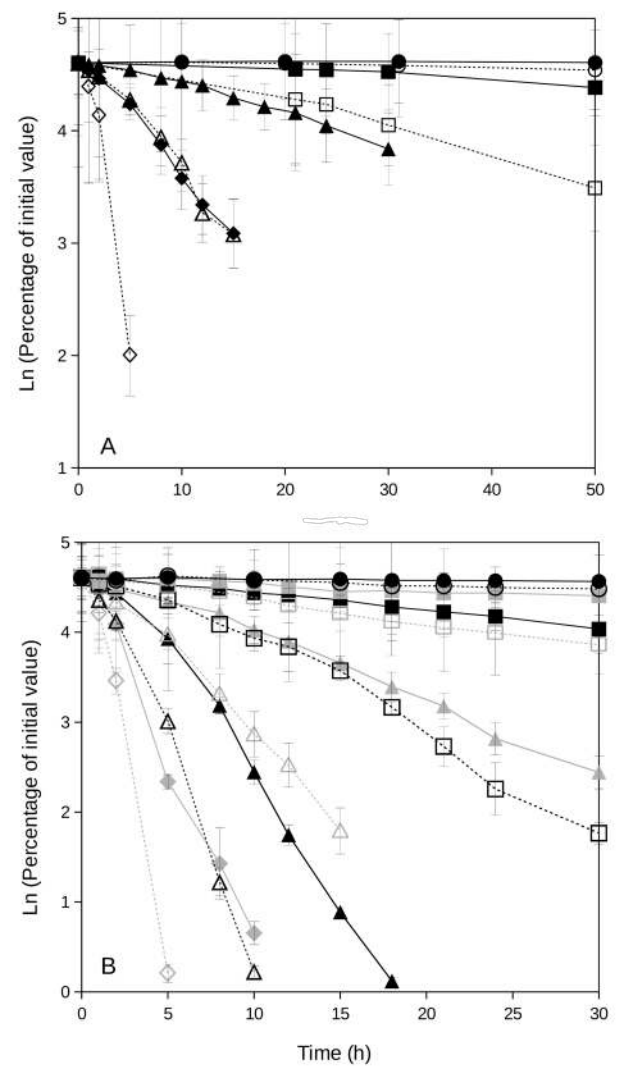
Figure 4. Relationships between NADH decay rates and viscosity for experiments performed at 20ºC (circles), 50ºC (squares), 70ºC (triangles) and 90ºC (diamonds). Different

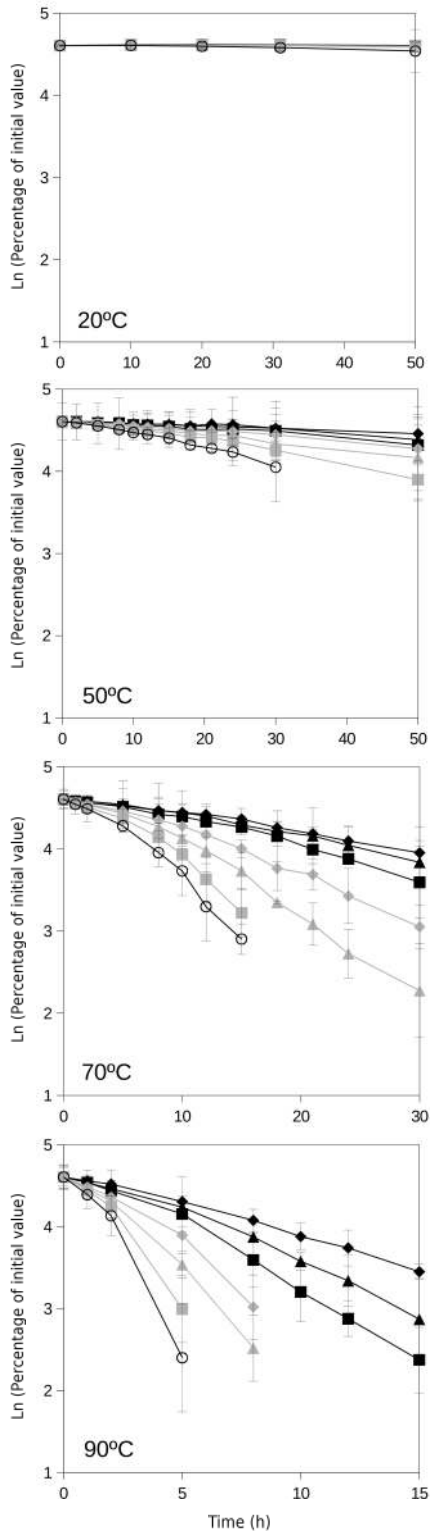
viscosities at a single temperature were obtained by using different concentrations of ethylene glycol (0%, 10%, 20%, 30%, 40%, 50% and 60%, final concentrations). Open symbols represent control solutions without ethylene glycol. Filled symbols of increasing darkness represent increasing ethylene glycol concentrations. The solutions with different concentrations of ethylene glycol assayed at the same temperature are linked by a continuous black line and they show the apparent trend of NADH stabilization at increasing viscosity. Solutions of equal composition are linked by a dotted line showing the effect of temperature on viscosity and NADH decay rate. Error bars are shown except when covered by filled symbols.

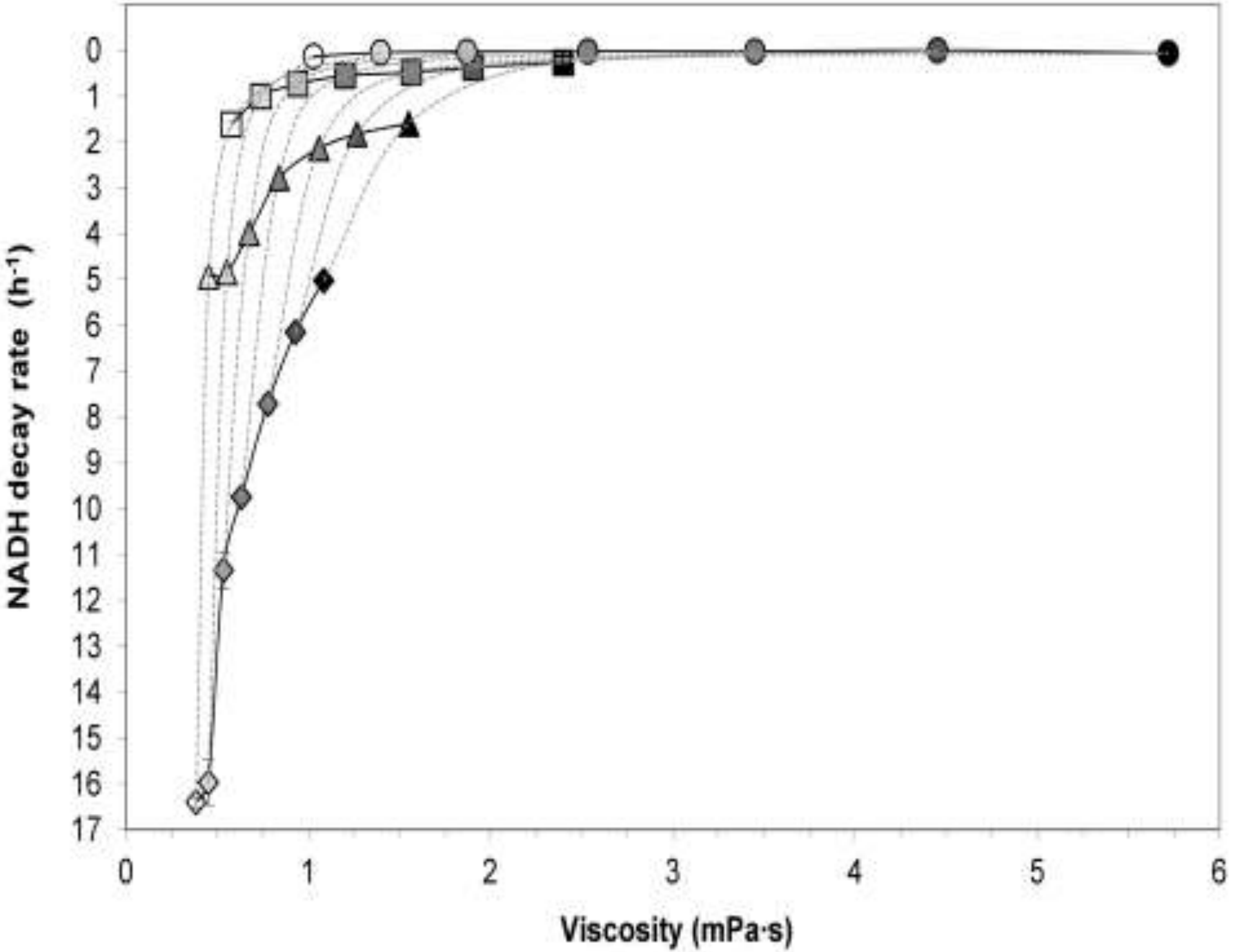
Figure 5. Relationship between cellular viscosity and growth temperature for several prokaryotes. *Escherichia coli* (open triangles), *Pseudomonas aeruginosa* (open circles), *Lactocccus lactis* (filled circles), *Geobacillus thermoglucosidiasus* (filled squares), *Fervidobacterium* sp. (filled triangles), *Pyrococcus furiosus* (open diamonds). Error bars are shown except when covered by filled symbols. A predictive non-linear curve (black continuous line) fitting the data points from species with filled symbols (*Lactococcus*, *Geobacillus*, *Fervidobacterium*) is presented. For comparison, the viscosity to temperature relationships of buffered-water (light grey dashed line) and 60% ethylene glycol solution (dark grey dashed line) are shown.

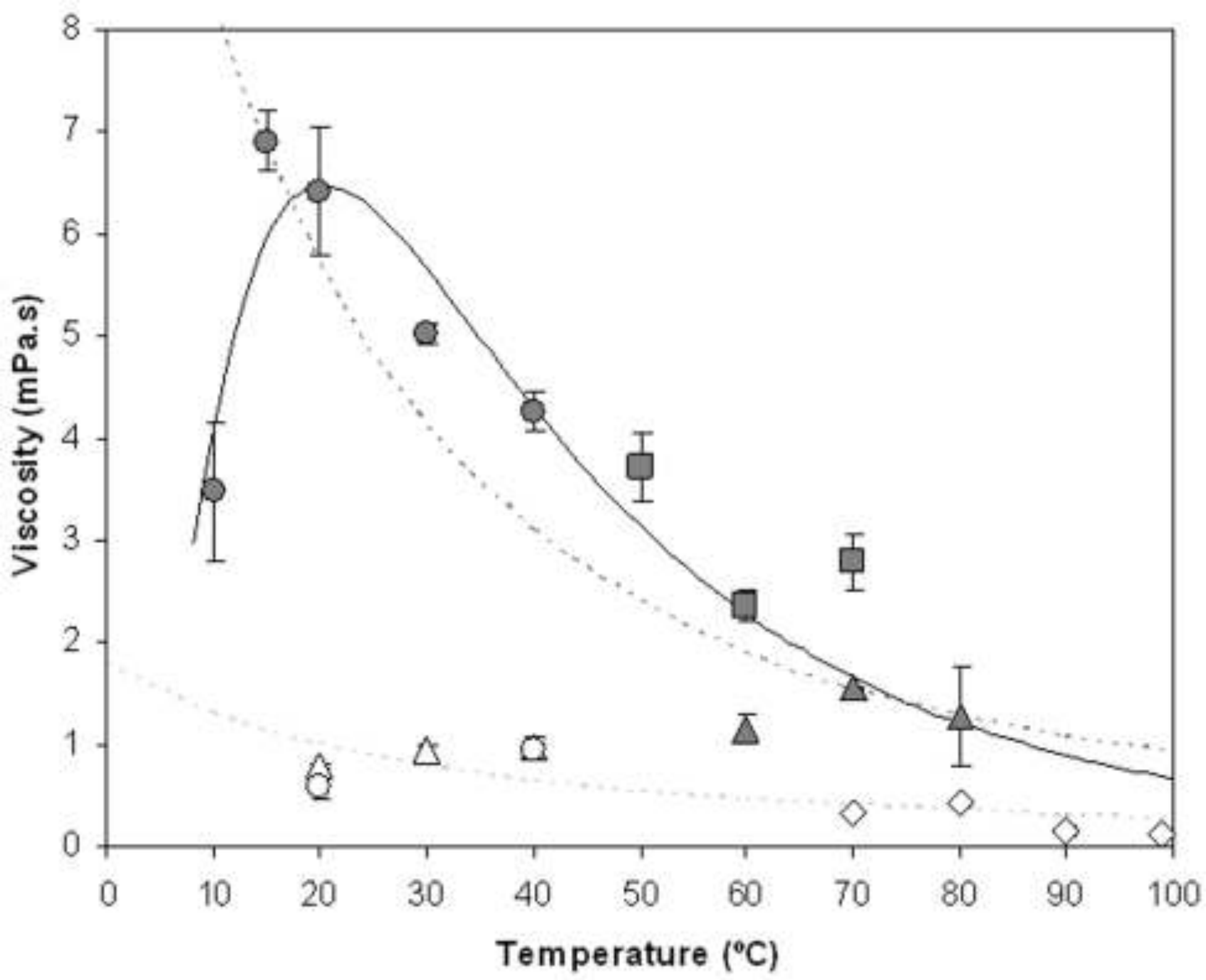
Figure 6. Fluorescence decay curves of RY3 for *Pyrococcus furiosus* cells grown at 80ºC (measured at 30ºC, upper figure, and at 80ºC, center figure) and *Lactococcus lactis* cells grown at 30ºC (measured at 30ºC, lower figure). Experimental data, fitted curves, residuals and estimated τ_{avg} values (ns) are shown for controls and cells.

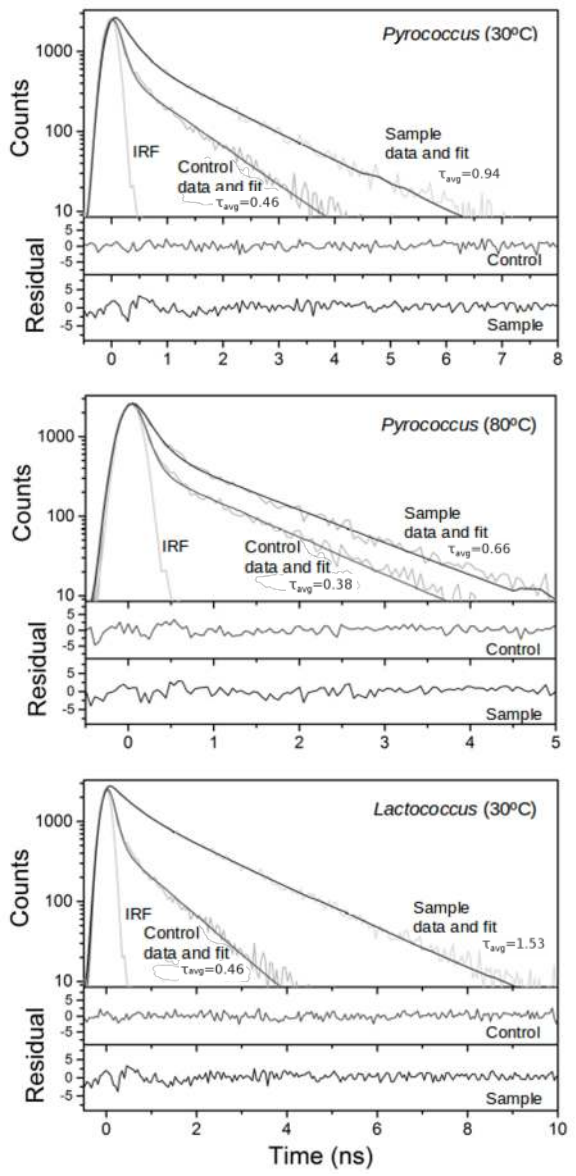

Viscosity (mPa·s)
0
1
2
3
4
5
6
7
8
20
30
40
50
60
70
80
90
Temperature (ºC)


Ln (Percentage of initial value)
A
B
Time (h)


Ln (Percentage of initial value)
20ºC
50ºC
70ºC
90ºC
Time (h)


NADH decay rate (h-1)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
0
1
2
3
4
5
6
Viscosity (mPa·s)


Viscosity (mPa.s)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
80
90
100
Temperature (°C)


Pyrococcus (30°C)
Counts
1000
100
10
IRF
Control
data and fit
τavg=0.46
Sample
data and fit
τavg=0.94
Residual
5
0
-5
Control
Sample
0
1
2
3
4
5
6
7
8
Pyrococcus (80°C)
Counts
1000
100
10
IRF
Control
data and fit
τavg=0.38
Sample
data and fit
τavg=0.66
Residual
5
0
-5
Control
Sample
0
1
2
3
4
5
Lactococcus (30°C)
Counts
1000
100
10
IRF
Control
data and fit
τavg=0.46
Sample
data and fit
τavg=1.53
Residual
5
0
-5
Control
Sample
0
2
4
6
8
10
Time (ns)