Detachment of *Listeria innocua* and *Pantoea agglomerans* from Cylinders of Agar and Potato Tissue under Conditions of Couette Flow.

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Abstract

Cylinders of raw potato or agar were contacted with suspensions of *Listeria innocua* and *Pantoea agglomerans* and then used as replacement rotors in a rheometer in order to investigate detachment under the influence of known shear forces. These shear forces were functions solely of the rotational speed of the rotor and the fluid (glycerol) in which the cylinders were caused to rotate. With this system surface shear forces ranging from 1.3 to 125 Pa could be generated corresponding to rotational speeds of 12.5 to 775 rpm. Under these conditions detachment phenomena were quite rapid with in most cases complete detachment being achieved over timescales of the order of 30 s. In general, lower shear forces were required to detach *L. innocua* from both agar and potato. For agar cylinders an applied shear force of only 1.3 Pa was sufficient to achieve 98 % detachment of *L. innocua* after 20 s. By contrast, relatively high shear forces were required to detach *P. agglomerans* particularly from potato; under an applied shear force of 2.8 Pa only 9.5 % detachment was achieved after 30 s. The results obtained at the highest shear forces studied here (125 Pa) with potato cylinders were suggestive of mass transfer into glycerol of one or more constituents present in potatoes that caused detached cells to aggregate causing an apparent

decrease in percentage detachment. The data obtained could be used as a basis for the rational design of washing processes for fresh ready to eat food products.

Keywords: Surface Shear Stress; Plant Tissue; Couette Flow; Bacterial Detachment; Washing Processes

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1. Introduction

The consumption of fresh ready-to-eat (RTE) salad vegetables and fruits has greatly increased over the last two decades (Li et al., 2001). These minimally processed foods are subject to contamination by a wide variety of microbial pathogens at every stage of their cultivation, harvesting and subsequent processing for retail (Mayer-Miebach et al., 2003). It is therefore not surprising that the growth in popularity of these commodities has been accompanied by an increase in incidences of foodborne diseases directly attributable to their consumption (De Roever, 1998).

The traditional method of decontaminating fresh salad products has been to wash them in chlorinated water. However, evidence has steadily been accumulating to show that the efficacy of this type of treatment is limited and may, for certain types of product e.g. sprouted shoots, be wholly inadequate (Gandhi and Matthews, 2003). Allied to this are concerns over the effects on human health of chlorine residuals (Kalmaz and Kalmaz, 1981) that have already resulted in its ban in certain countries.

Taken together these factors would appear to spell the demise of this form of decontamination treatment in the foreseeable future.

It is unlikely that any single treatment will replace washing with chlorine throughout the entire RTE food sector. A number of alternatives have been proposed and these include the use of chlorine dioxide gas (Han et al., 2000), heat shock coupled with calcium lactate (Rico et al., 200&), hydrogen peroxide (Ukuku, 2004) and, UV and ozone treatment (Bialka et al., 2008). However, all such treatments are more costly than chlorine washing and most have yet to clear the final hurdle of consumer acceptance.

Recent work (Wei et al., 2005) has shown that significant decreases in microbial viability can be achieved simply by the use of acidified water at 50° C. There are therefore, distinct advantages in ensuring that the maximum level of decontamination is achieved by water washing before resorting to any of the newer decontamination techniques cited above. The objective of washing is to remove dirt, pesticide residues and micro-organisms (Baur et al., 2005) and this is typically achieved using flumes, and a wide variety of this type of equipment is commercially available (e.g. Rodriguez, 1999). In virtually all cases these designs have been arrived at largely by heuristics. However, it remains possible that if a systematic approach were taken to the design of washing processes, novel configurations might emerge. This approach would firstly require knowledge of the shear forces required to physically detach the microflora associated with a particular type of produce. It would also rely on an appreciation of the effects on the produce of exerting such forces (Hassan and Frank, 2003) in order to minimise losses through physical damage to the plant tissue.

As a first step towards this process, we present a novel and precise method of estimating the effects of shear forces on the detachment of bacteria from solids. We did this by generating a known shear force at the surface of the solid – itself in the form of a cylinder – by causing it to rotate inside a slightly larger hollow cylinder filled with a viscous liquid (glycerol). The velocity distribution generated in the annular gap between the two cylinders is referred to as 'Couette flow'. This was achieved in practice by using a commercial rheometer in which the rotational element or 'rotor' was replaced by one made from materials of interest. These were agar, chosen here to represent a model food compound (Midelet and Carpentier, 2004), and raw potato. These experiments were conducted using *Listeria innocua* and *Pantoea agglomerans*. The former is widely regarded to be a surrogate for the pathogen *L. monocytogenes* the causative agent of listeriosis (Perni et al., 2006) and frequently associated with fresh produce (Beuchat, 1995). *P. agglomerans* is a biofilm-forming plant pathogen that is also known to colonise fresh produce (Brocklehurst et al., 1987).

2. Materials and methods

2.1 Detachment studies

These studies were conducted using a rotational rheometer (Viscotester VT 550, Haake GmbH, Karlsruhe, Germany) comprising a stainless steel cylindrical rotational element or 'rotor' and a static element or 'cup'. We substituted the rotor supplied with the rheometer by ones made either of raw potato or agar. In either case, the cylinders

had to be generated consistently to precise dimensions (dia. 20 mm; length 60 mm) and in such a way that when positioned in the cup, they were concentric with one another and in alignment with the axis of the instrument.

2.2 Production of Potato and Agar Cores

A specially fabricated stainless steel prong comprising three hollow tines at one end and a cylindrical shouldered shaft at the other was employed first. The shaft had been machined in such a way as to enable it to be coupled directly to the motor drive of the rheometer. The prong was pushed by hand into the flat surface of a potato prepared by slicing through a potato with a knife (Figure 1a).

A coring device, consisting of a stainless steel tube (o.d. 21 mm, length 200 mm) sharpened at one end and containing inside it a sliding internal guide, was then located directly over the prong so that the guide fitted onto the shouldered cylindrical shaft (Figure 1b). The corer was forced into the potato until it had completely penetrated it, at which point it was withdrawn yielding a cylinder which was cut to the requisite length (60 mm) with a scalpel to yield the mounted core shown in Fig. 1c

Prior to use, the surface of the potato was decontaminated by spraying with a 70 % (v/v) aqueous ethanol solution and then dried in a laminar flow cabinet for 30 minutes, in addition, all components were flame-sterilised.

In order to produce agar cylinders, the prong was placed vertically into approximately 300 ml of molten TSA (Tryptone Soy Agar, Oxoid) supplemented with 3 % (w/v) agar no. 3 (Oxoid) in a sterilised glass beaker so that the tines were completely

submerged. Once the agar had set, the coring procedure described above was put into operation.

Once produced, the cores were lightly sprayed with 70 % (v/v) aqueous alcohol and then dried for 30 minutes in a laminar flow cabinet prior to immersion in bacterial suspension.

2.3 Micro-organisms and cultivation

L. innocua (ATCC 33090) and *P. agglomerans* (isolated from lettuce and kindly donated by Professor T. F. Brocklehurst of the Institute of Food Research, Norwich, UK) were both maintained on TSA agar slopes at 4°C. Sterile TSB (100 ml), contained in 150 ml Duran bottles with specially modified tops enabling the cores to be suspended vertically whilst in contact with its contents, were inoculated with a loopful of bacteria previously grown for 24 hours on TSA plates incubated at 30 °C in the case of *L. innocua* and 20 °C for *P. agglomerans*. The broths were incubated statically at the same temperatures quoted above for 24 hours.

A core equilibrated at 30 °C in the case of *L. innocua* and 20 °C for *P. agglomerans for 30 min* prepared as described above was immersed in cell suspension for 2 hours at the relevant growth temperature. After removal, the core was washed twice by immersing it in succession into each of two 150 ml Duran bottles containing 100 ml of sterile PBS before attaching to the rheometer or priorto conducting counts. The mean initial counts (in CFU/cm^2) for *L. innocua* were 9.3 x10^6 on potato and 3.0 x 10^6 on agar. The counts for *P. agglomerans* were 1.47 x 10^7 and 1.1 x 10^7 respectively.

For detachment studies the rheometer cup was filled with 100 ml of sterile glycerol and the core gently lowered into it. Samples of glycerol (100 μl) were taken from approximately the same position in the gap between the core and wall of the cup and plated onto TSA plates after appropriate dilution in PBS. Plates were incubated for 24 hours at 30 °C in case of *L. innocua* and 20 °C for *P. agglomerans* before counting.

We present our results in terms of 'percent detachment' and in order to do this obtained estimates of the cell numbers adhering to the cores immediately after washing, as described above, by placing a core in a plastic bag with 50 ml of sterile PBS and processing in a Stomacher (Model 400, Seward Ltd., Thetford, UK) for 2 minutes on 'normal' setting and plating out on TSA plates.

The percentage detachment at any time t was calculated thus:-

$$percent\ \det achment = \left\{ \frac{cell\ concentration\ in\ glycerol(t)^{1}\ x\ total\ vol.\ of\ \ glycerol}{surface\ area\ of\ \ core} \Big/ surface\ conc.\ of\ \ cells\ at\ t = 0^{2} \right\} x100$$

1 obtained directly from counts and expressed as CFU/ml
2 expressed as CFU/cm^2

All experiments were repeated using three independently grown cultures.

2.4 Calculation of surface shear

The relationship between shear stress and shear rate is given by:

$$\tau = \eta\gamma$$

where:

τ = shear stress (Pa)

γ = shear rate (s^{-1})

η = viscosity (Pa s)

and

$$\gamma = nM$$
$$\tau = fM_d$$

where:

n = rotor speed (rpm)

M_d = torque (N cm)

M, f = dimensionless instrument-dependent system parameters, 0.245 and 471.6 respectively.

2.6 Statistical Methods

The cell counts obtained at each exposure time at various shears were compared using the ANOVA test followed post hoc by Tukey's test for individual pair of data sets. Statistical analyses were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA)

3. Results and discussion

The procedures developed here enabled cores to be consistently produced within close physical tolerances, and this coupled with the electronically controlled speed at which the cores were rotated, meant that the shear forces at the surface were maintained constant over time and were solely functions of the rotational speed. Figure 2 displays the theoretical relationship between rotor speed, shear rate and shear stress for a conventional steel rotor rotating in glycerol (a Newtonian fluid) and that obtained by replacing the steel rotor with one made of raw potato of identical dimensions. The data was obtained by taking instrument readings over the range 12.5 to 775 rpm corresponding to shears forces between 1.3 and 125 Pa. The close agreement between the two enables us to quote below with high precision the shear forces acting on the surface of the agar and potato cores.

Figure 3a shows the percentage detachment of *L. innocua* from agar cores. The point shown at time zero in this and subsequent figures, and indicated by an 'x', was obtained by allowing cells to attach to the core in the normal way and then immersing it at a steady rate into the cup containing the glycerol whereupon the latter was immediately sampled without rotation of the core. This was done to allow an estimate to be made of cell detachment caused by passage of the core through an air-liquid interface. At the lowest shear force of 1.3 Pa, a gradual detachment of cells occurs until 20 s had elapsed when a value of 98 % detachment was achieved, and after which there was apparently no further loss of cells. Detachment at all shear forces above this value (i.e. 2.8, 11.8, 125 Pa) was very much more rapid and all the profiles are very similar. By 30 s approximately 100 % detachment had occurred regardless of

224 applied shear force.The results for 1.3 Pa were significantly different (P<0.5) from
225 those at 2.8 and 11.8 Pa at 5 and 10 sec. However, after 20 and 30 sec there were no
226 significant differences between all three applied stresses. There were no significant
227 differences in cell detachments brought about by shear stresses of 2.8 and 11.8 Pa
228
229
230 The results obtained for agar cores with *P. agglomerans* are shown in Figure 3b. For
231 an applied shear force of 11.8 Pa the profile displays a slight peak at 10 s but the
232 detachment remains low at below 10 %. At the higher shear force of 125 Pa a steady
233 loss of cells is observed and at 30 s 85 % detachment was attained. There were no
234 significant differences in cell detachments after 5 sec. After 10, 20 and 30 the counts
235 at 11.8 Pa were significantly different from those at 125 Pa
236
237
238 The detachment of *L. innocua* from potato cores (Figure 4a) shows clear differences
239 at shear forces of 1.3 and 2.8 Pa. At a shear force of 1.3 Pa the detachment achieved
240 after 30 s was 36 % whereas at 2.8 Pa it was 106 %. The profile obtained at 125 Pa
241 appears anomalous: detachment was initially rapid and after 5s had reached a value of
242 56 %, thereafter, an apparent decrease was observed with detachment remaining
243 approximately constant at 47 %. The value of detachment at 30 s was actually lower
244 than was achieved under the influence of a shear force of 2.8 Pa even though the shear
245 force acting at the surface of the core was over 4 times greater. There were no
246 significant differences between the number of cells detached at all three applied shear
247 stresses at 10, 20 and 30 sec. Cell counts at 5 sec at 1.3 Pa were significantly different

248 from those at 2.8 and 11.8. There were, however, no significant differences in the
249 number of cells shed at the latter two applied stresses.
250
251
252 The detachment profiles for *P. agglomerans* from potato (Figure 4b) show the rate at
253 2.8 Pa to be relatively low reaching a value of 9 % after 30 s. When the shear force
254 was increased to 11.8 Pa a peak detachment of 18 % was achieved at 20 s and this is
255 followed by a decline to a value (7 %) which is close to that achieved at 2.8 Pa. The
256 results at 125 Pa also reveal a marked anomaly with an initial value of 81 % which
257 actually decreases quite markedly to 13 % by 30 s. All counts were statistically
258 different at 5, 10 and 20 sec. but not at 30 sec.
259
260
261
262 The procedures developed here enabled cores to be consistently produced within close
263 physical tolerances, and this coupled with the electronically controlled speed at which
264 the cores were rotated, meant that the shear forces at the surface were maintained
265 constant over time and were solely functions of the rotational speed. Moreover, these
266 were mathematically predictable with a high degree of precision. However, it was
267 necessary to account for the shear forces generated when an object is transferred
268 across an air-liquid interface as in our case inevitably occurred when the cores were
269 lowered into the glycerol. Meinders et al. (1992) have argued that these forces can be
270 significant and our results show that this action did indeed result in measurable
271 detachment. The shear forces that were generated at the surface of the potato and agar
272 as a result of rotation in glycerol were too low to cause deformation of the cores

273 themselves as confirmed by measurements made by Alvarez and Canet (1998) for
274 potato and Barrangou et al. (2006) for agar.
275
276 The results presented here show that *P. agglomerans* required a greater force to
277 detach it from the surfaces of both potato and agar than did *L. innocua*. In the case of
278 potato this was not unexpected given that the *P. agglomerans* is not only a plant
279 pathogen, but a known biofilm former and therefore likely to be specially adapted to
280 colonize plant tissue. However, studies of the complex interactions that occur between
281 micro-organisms and plant tissues are primarily confined to the surfaces of leaves
282 (Frank, 2001) and no specific biochemical studies on the particular combination of *P.*
283 *agglomerans* – potato currently appear to exist. Similar findings were reported by
284 Garrood et al. (2004) who compared, amongst other organisms, the attachment and
285 detachment of *P. agglomerans* and *L. monocytogenes* to discs of raw potato. Although
286 these workers did not attempt to estimate shear forces, they modelled the processes of
287 detachment on a probabilistic basis.
288
289 The method employed here to monitor detachment was based on the detection of
290 viable bacteria that had been shed from the surface of the cores into the glycerol.
291 Therefore, any apparent declines in detachment – as were observed with *P.*
292 *agglomerans* from potato at high shears (Figure 4b) – are suggestive of other
293 phenomena acting on detached cells in suspension. The fate of detached cells may
294 have been affected by a number of factors (both chemical and physical) that, although
295 were not directly related to shear, are a consequence of it.
296

297 One possibility is loss of viability whilst in suspension. It is highly unlikely that
298 glycerol itself would have contributed to this. Although Saegeman et al. (2007)
299 investigated the effect of concentrated glycerol solutions (85 %) on the viability of
300 both Gram positive and Gram negative bacteria, the times at which they achieved
301 reductions were of the order of days, rather than the short contact times measured in
302 seconds employed in this work when detached cells were actually in contact with
303 glycerol prior to plating onto agar. Another possibility is the release of toxic
304 compounds from the potato tissue. Potato tubers have been shown to contain a diverse
305 range of constitutive and inducible anti-microbial compounds that include peptides,
306 enzymes and phytoalexins (Berrocal-Lobo et al., 2002; Guevara et al., 2002; Lopez-
307 Solanilla et al., 2003). At high surface shears rapid mass transfer of such compounds
308 could be expected to take place followed by efficient dissipation throughout the mass
309 of glycerol present in the cup. However, given the short contact times and low
310 concentrations, any such compounds would need to have considerably higher activity
311 than any that have so far been isolated from potatoes by previous workers.
312 A more likely explanation for the apparent decrease in viability of detached cells are
313 purely physical phenomena which are faster acting. Under the influence of high
314 surface shears, starch and possibly other constituents of potato, could have been
315 caused to be shed into the glycerol along with bacteria. Starch has been used to bring
316 about the efficient flocculation of bacterial cells in order to harvest them (Ferenci and
317 Lee, 1991). The apparent declines in viability observed here could be explained by
318 only a low degree of flocculation or cell aggregation occurring due to starch or other
319 constituents of potato.
320

There are obvious limitations in employing an instrument for a purpose for which it was not designed: the data presented here, in common with that of others (Garrood et al., 2004) reveals that micro-organism - surface interactions are rapid, and therefore correspondingly rapid sampling is called for. We encountered sampling difficulties primarily because of the viscosity of the glycerol, and it was not possible to obtain a sample of annular fluid earlier than 5 sec. after rotation of the core was initiated. Furthermore, the dimensions of the annulus between the rotor and the edge of the cup was only 13 mm. Every effort was made to sample from the mid point of the annulus but even this was rather imprecise. We believe that these difficulties can be overcome and indeed, are in the process of designing and fabricating an improved device that will feature automated sampling coupled to a fraction collector that will enable more extensive studies to be performed including that of leafy produce such as lettuce.

4. Conclusions

The equipment described here was successfully used to obtain quantitative data that could ultimately be used in the rational design of washing processes for microbially decontaminating fresh produce. The advantages of employing water rather than solutions of chemical agents are obvious however apparently benign such agents may prove to be. Even if recourse has to be made to such agents, it would seem logical that the design of water-washing processes should be made as efficacious as possible so that any subsequent chemical treatment can be brief and employ only dilute concentrations.

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Figure Legends

Figure 1. Procedure for Preparing Potato Cores

a) Prong inserted into cut potato. b) Coring device located over prong. c) Completed core.

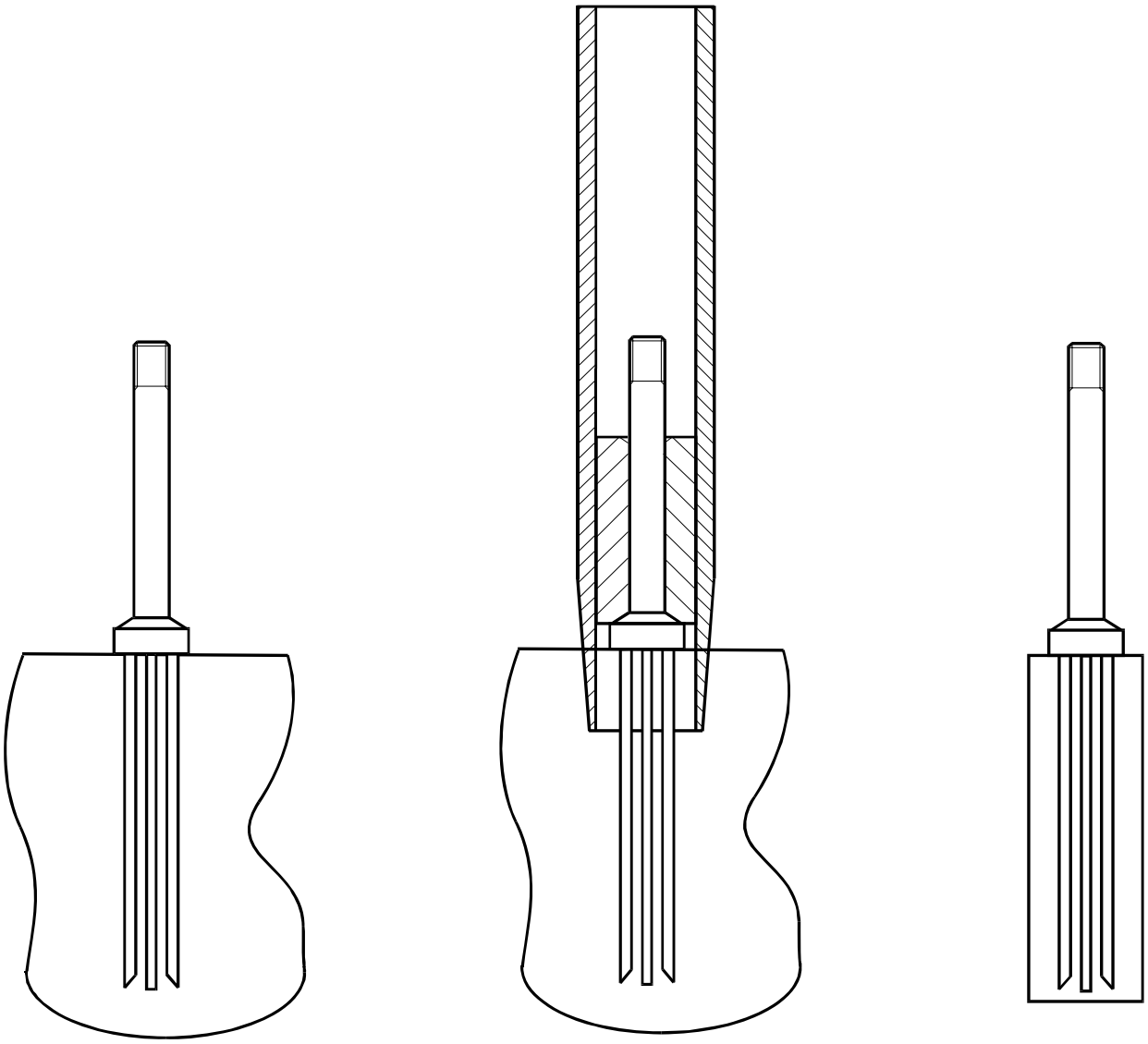
Figure 2. Dependence of Shear Stress at Rotor surface on Speed and Shear Rate. Solid line denotes theoretical relationship. (●) Experimentally obtained values for potato cores.

Figure 3. Detachment of *L. innocua* (a) and *P. agglomerans* (b) from Agar Cores

× initial detachment ● 1.3 Pa ■ 2.8 Pa Δ 11.8 Pa ◊ 125 Pa

Figure 4. Detachment of *L. innocua* (a) and *P. agglomerans* (b) from Potato Cores

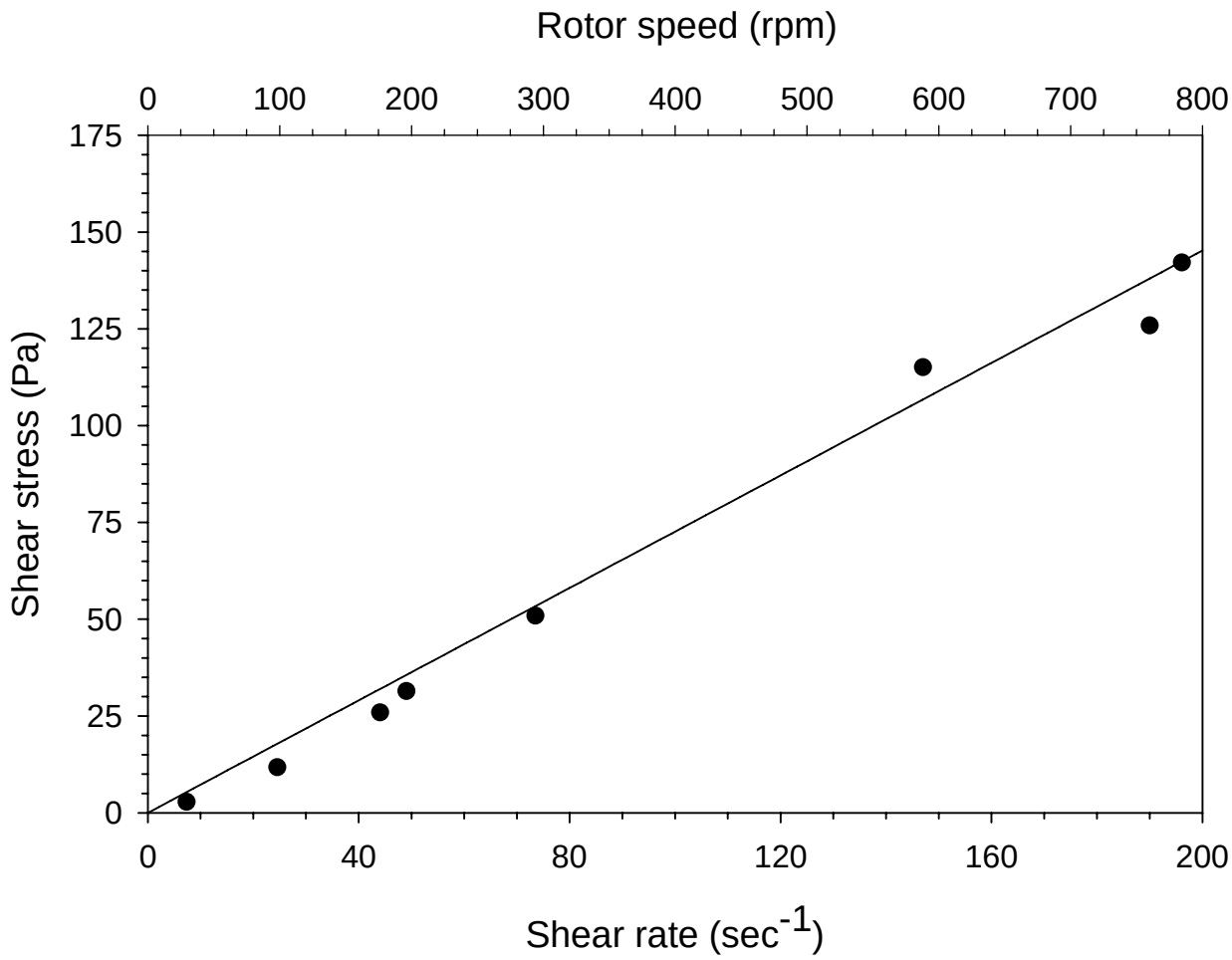
× initial detachment ● 1.3 Pa ■ 2.8 Pa Δ 11.8 Pa ◊ 125 Pa



a b c

481

482 Figure 1



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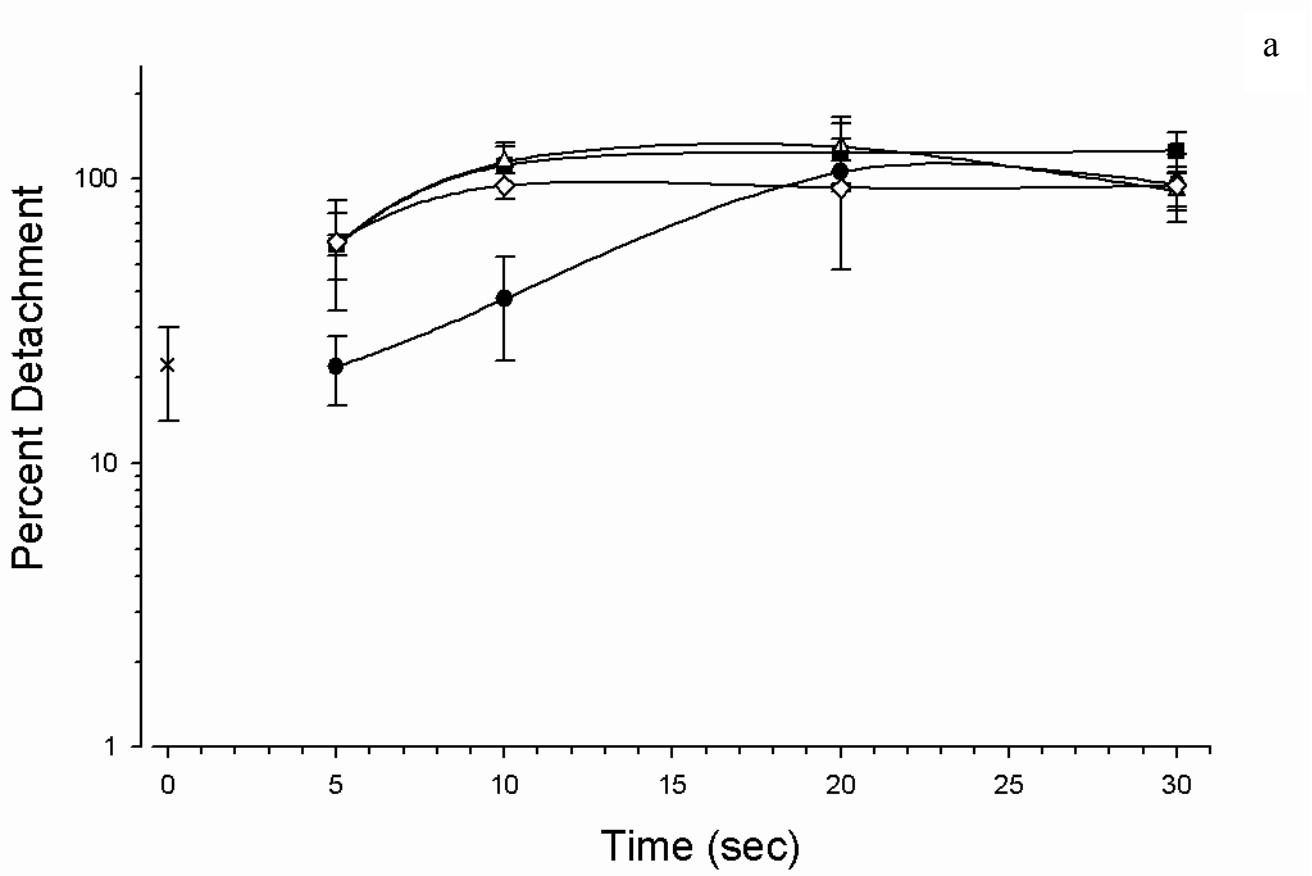
485 Figure 2


a
Percent Detachment
100
10
1
0
5
10
15
20
25
30
Time (sec)

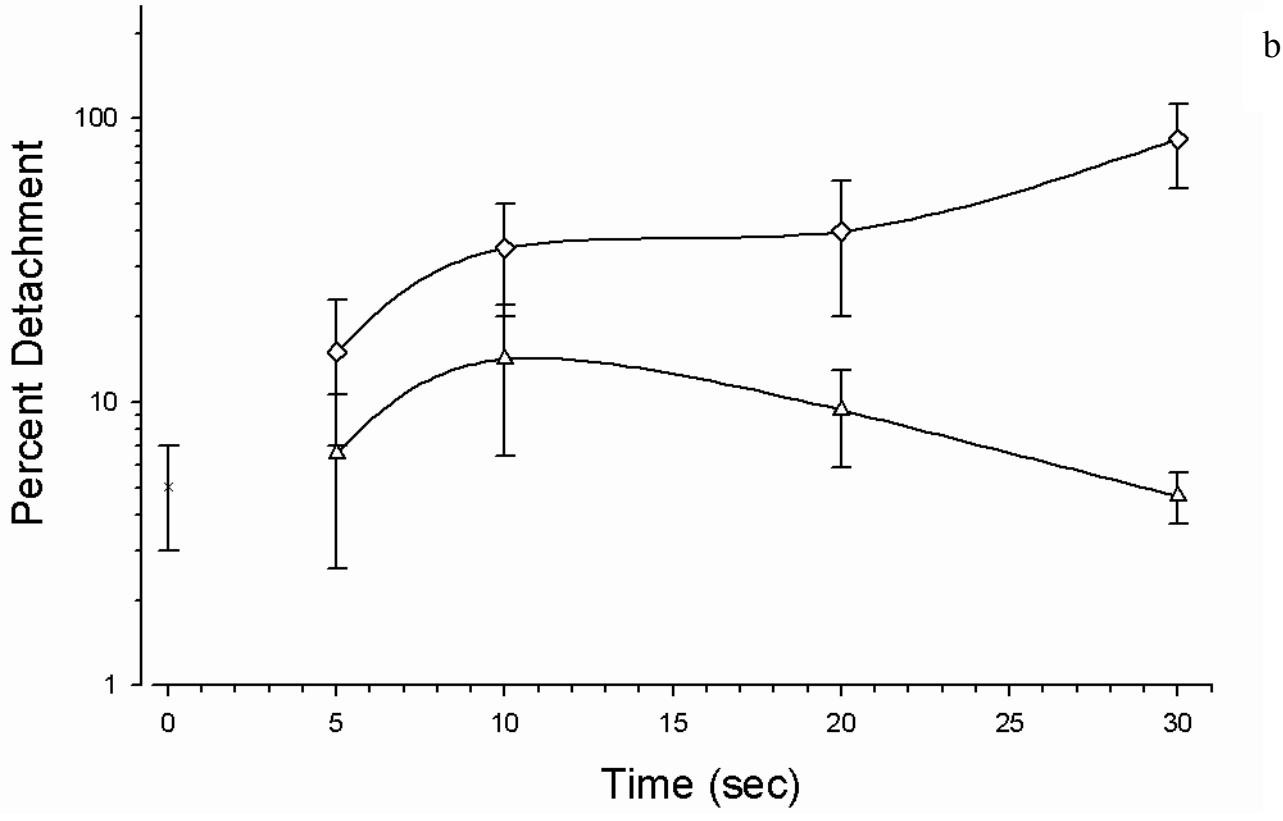
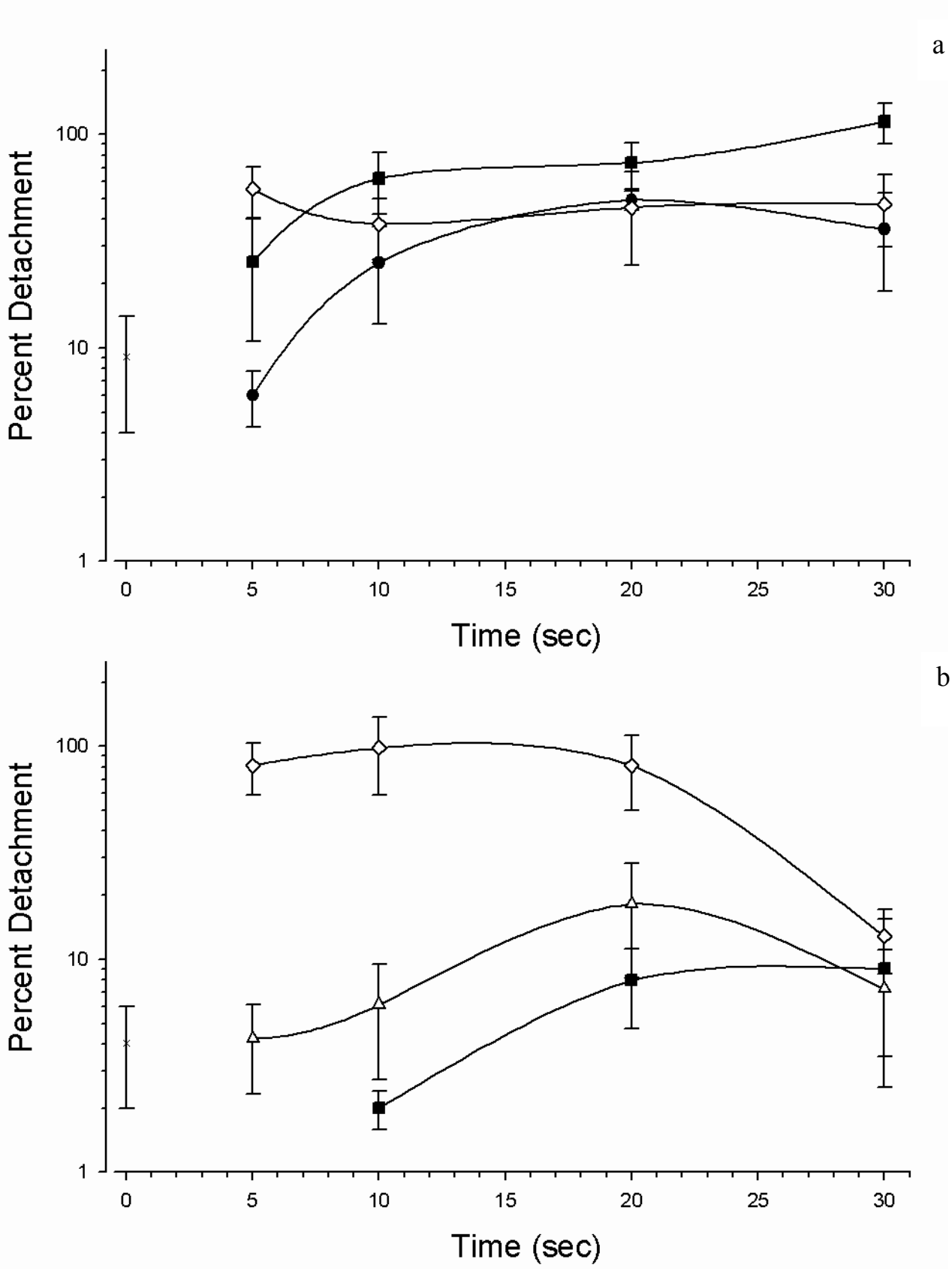

b
Percent Detachment
100
10
1
0
5
10
15
20
25
30
Time (sec)

Figure 3

490



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493 Figure 4

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a

b