EFFECT OF TEMPERATURE AND MODIFIED ATMOSPHERE PACKAGING ON THE GROWTH OF *LISTERIA MONOCYTOGENES* IN COLESLAW

by

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ABSTRACT

The growth of *L. monocytogenes* in modified atmosphere packages and ambient air packages was studied at refrigeration temperature, 3°C, and at the slightly higher temperature of 8°C, since it is possible that at some point during distribution or consumer handling temperature abuse will occur. Three levels of *L. monocytogenes* inoculum were used: non-inoculated, 10^4/g and 10^5/g. Modified atmosphere did not affect the growth of *L. monocytogenes*. The higher temperature resulted in a higher level of growth of *Listeria* in the coleslaw but obvious indication of spoilage (odour, discoloration and/or package swelling) was not present before populations of *L. monocytogenes* had reached 10^6 cfu/g. The length of storage has a significant effect ($p \leq 0.001$) on counts of both total bacteria and *L. monocytogenes*. Inoculum levels did not have a significant effect on total bacterial counts overall. In the second phase of this study, the ability of *L. monocytogenes* to utilize 95 different carbon sources at 2 different temperatures was examined by monitoring O.D. readings over time using a Biolog™ MicroPlate. *L. monocytogenes* metabolised 33 carbon sources at 30°C and only 21 at 8°C.

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INTRODUCTION

Listeria monocytogenes, first described in 1926 (103), has recently emerged as a bacterium of considerable public health significance. Prior to the 1980's, listeriosis, the disease caused by *L. monocytogenes*, was primarily of veterinary concern, where it was associated with abortions and encephalitis, most commonly in sheep and cattle (31). Interestingly, evidence indicates that veterinary listeriosis was frequently foodborne. Outbreaks were described in rabbits, goats and chinchillas. Chinchilla farms were affected in one outbreak in Nova Scotia which was attributed to feeding a new batch of meal containing beet pulp (31).

Although *L. monocytogenes* is widely distributed in nature (44, 100, 121), the number of outbreaks have been low. Case fatality is high in susceptible individuals; approximately one-third of cases have resulted in deaths or stillbirths (1). Listeriosis is characterized by flu-like symptoms such as vomiting, nausea, cramps, diarrhoea and constipation and, in untreated cases, serious complications such as meningitis/encephalitis and/or septicemia (25, 87). Stillbirths, abortions, and premature birth of an infected infant can result from only a mild illness in pregnant women. Therefore those at particular risk to listeriosis include pregnant women and their unborn children but also patients undergoing chemotherapy, alcoholics, drug abusers, diabetics and AIDS patients (25).

L. monocytogenes is a small, motile (at 20 to 25°C), Gram positive, catalase positive, non-spore forming and noncapsulating rod which is aerobic to microaerophilic (100). *L.*

monocytogenes, when observed microscopically, appears as short diphtheroid-like rods measuring 1.0-2.0 μm x 0.5 μm (101). *L. monocytogenes* occurs widely in the environment. *Listeria* can be found in many species of fish, fowl and over forty other animals and is common in mud, dust, silage and various waste products (13, 87). *Listeria* can grow at a variety of temperatures, from 1°C to 45°C, and thus can survive and grow in foods kept at refrigeration temperatures (39). Three characteristics allow *L. monocytogenes* to survive and grow in foods: wide temperature range tolerance, psychrotrophic nature, and resistance to high salt concentrations (1, 39, 101, 102).

L. monocytogenes should be considered as an environmental microbe whose primary means of transmission to humans is through contamination of foodstuff at any point in the food chain from its source to the home kitchen (1, 13, 87). Food appears to be the major means of zoonotic transmission of listeriosis. The definition of zoonosis is "an infection or an infectious disease transmissible under natural conditions from vertebrate animals to man" (101). Circumstances of the outbreaks and the implicated foods support the concept of zoonotic foodborne transmission of listeriosis (101), especially by milk and milk products and raw vegetables.

Cabbage and other raw vegetables have been identified as potential vectors of listeriosis (13, 48, 61). The consumption of coleslaw prepared from contaminated cabbage was linked to a large outbreak of listeriosis in Nova Scotia in 1981. Thirty-four cases of prenatal and 7 cases of adult listeriosis were reported (43, 101). This listeriosis outbreak resulted in 18 fatalities (101).

1.1 Research objective

The primary focus of this thesis was to investigate the survival and growth of *L. monocytogenes* in fresh packaged coleslaw. The ability of *L. monocytogenes* to survive longer under adverse environmental conditions, unlike most other non-spore forming bacteria, and particularly its ability to grow at refrigeration temperatures, may lead to illness and death in humans (13). *L. monocytogenes* may be more heat tolerant (13) and acid tolerant (27) than previously speculated, therefore making this organism a threat to the food industry. Therefore, *Listeria's* survival and growth are an important consideration for pre-packed coleslaw, particularly when prepared salads can be subjected to long holding times in refrigerated display cabinets (48).

This study characterizes the growth of *L. monocytogenes* Scott A in coleslaw at refrigeration temperatures that could result from inadequate cooling during distribution or in retail display.

Phase II of this research examined the effect of refrigeration temperature on the growth of a pure strain of *L. monocytogenes* compared to growth at a more optimum temperature. The temperature of chilled foods is an important consideration for the shelf life of a product such as coleslaw in a production and distribution chain.

LITERATURE REVIEW

2.1 Historical background

Food has been implicated as the vehicle for transmission of human listeriosis. The outbreaks are often fatal (64), therefore emphasizing the need for characterization of the growth and survival of *L. monocytogenes* in food. Its wide distribution in nature and its ability to survive for long periods under adverse conditions as well as grow at refrigerator temperatures makes *L. monocytogenes* a potential threat to ready-to-eat products like coleslaw.

Until 1950 approximately 70 cases of human listeriosis had been documented (98). The first outbreak to be shown conclusively to be caused by food consumption was in Nova Scotia in 1981 (26, 35, 39, 98).

2.1.1 Outbreaks of the disease

The first confirmed human listeriosis case in Canada was recorded in 1951 when a pregnant woman who had emigrated from Russia was diagnosed with listeriosis (32, 39). A later survey based on records maintained at the Laboratory Centre for Disease Control, Ottawa showed 101 cases detected over a 21-year period from 1951 to January 1972 in 9 of 10 provinces (31, 32).

A review of the records at Vancouver General Hospital over a 15-year period, from 1965 to 1979, showed 22 cases of confirmed listeriosis. The overall mortality was 30% and the most frequent serotypes involved were 4b followed by 1 and 1b (31, 32).

No major outbreaks have been documented in Canada with the exception of one occurring in 1981 in Nova Scotia. The Nova Scotian epidemic was between March and September 1981. There were 41 cases, 34 perinatal (either infected fetus or infected newborn babies) and 7 adult. Of those infected, 15 neonates and 2 adults died. *L. monocytogenes* serotype 4b was isolated from coleslaw from a patient's refrigerator. The same serotype was found in unopened packages from the manufacturing plant, and from cabbage grown on land fertilized by sheep manure. Three sheep from the farmer's flock died from listeriosis (98). The cabbage was harvested in October of 1980 and stored during the winter and early spring in cold storage, ideal conditions for multiplication of a small inoculum of *L. monocytogenes* (31).

There have been two documented outbreaks in the United States (98), two in the United Kingdom (98) and one each in Switzerland (68), Australia and France. In 1983, in Massachusetts, an outbreak affected 49 people (42 adults and 7 infants) and 14 patients died (37). The case was associated with 2% or whole milk from a single plant. All milk had been pasteurized in excess of the legal requirements. One farm supplying milk to the plant was shown to harbour *L. monocytogenes* of the same serotype but of different phage type from the epidemic strain (98).

Fleming *et al.*. (46) postulated that *L. monocytogenes* present in large numbers in the raw milk had survived pasteurization and multiplied at subsequent refrigeration storage temperatures of the pasteurized product. The organism may be able to survive pasteurization because it is ingested by leucocytes and then located inside the somatic cell which provides some form of protection (39).

Much scientific research has been devoted to the thermal survival of *L. monocytogenes*. Inspection of milk from a processing plant in Spain revealed that *L. monocytogenes* was present in 21% of pasteurized (78°C, 15 sec.) milk samples (53). Research by Knabel *et al.*(76) supported the possible survival of low levels of *L. monocytogenes* during high temperature, short time pasteurization by recovering injured cells on selective media under anaerobic conditions. Farber's (78) work at the Food and Drug Administration reported no survival and the FDA research also could not recover *L. monocytogenes* in 23 milk pasteurization trials (78). Full-scale pasteurization studies by the FDA could not detect *Listeria* in pasteurized milk using four different assay methods (84).

After the Massachusetts epidemic in 1983, there was an outbreak of listeriosis in California in 1985 with 142 cases in which 29 people died (37, 105). The likely source was Mexican-style fresh cheese, where serotype 4b was isolated as well as from the processing plant. Again, the isolation of *L. monocytogenes* suggested ineffective pasteurization or contamination of raw milk (98).

Two outbreaks have occurred in the United Kingdom, one in 1986 with imported soft cheese and the other in 1988 with goat's milk cheese. The isolated level of *L. monocytogenes*

in the cheese was 10^8/g (98).

In 1987, there were 31 cases of human listeriosis in Vacherin cheese in Switzerland (68); in 1991, one case in smoked mussels in Tasmania; and in 1992, 279 cases in jellied pork tongue in France (67).

2.1.2 Transmission of *L. monocytogenes*

The recent listeriosis outbreaks have resulted in recognition of *L. monocytogenes* as a foodborne pathogen (13, 95, 101). A variety of animals can serve as hosts for *L. monocytogenes.* The organism can be isolated from such animals as sheep, poultry, cattle, horses, pigs, moose, dogs, cats, as well as humans without symptoms (13, 101). The cycle of infection for *L. monocytogenes* as proposed by R.E. Bracket (1988) is illustrated in figure 1 (13).

Surveys have been done to determine the extent to which *L. monocytogenes* is present in various kinds of foods. In 1968, Welshimer (117) sampled old vegetation from 12 farms in Virginia for the occurrence of *L. monocytogenes* and isolated 8 strains, of which only 2 strain types were pathogenic. In 1989, Lacey and Kerr (79) found that 25% of the 64 cool/chill products tested were contaminated with *L. monocytogenes.*

Tiwari and Aldenrath (111) sampled 598 food products and environmental swabs, from the province of Alberta, for *L. monocytogenes* to assess the extent of contamination in food products. Five of 15 raw milk samples from dairy plant holding tanks were positive for

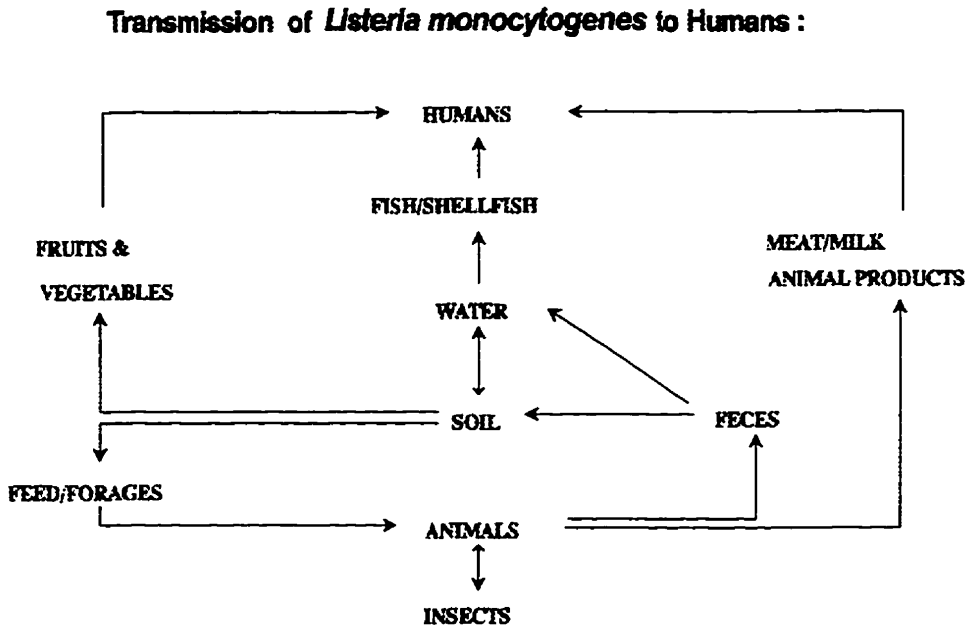

Figure 1. Hypothesized Cycle of Infection for *Listeria monocytogenes* proposed by Brackett (1988).

Listeria and 4 of these were *L. monocytogenes.* Testing of raw meat revealed that 44.4% were positive for *L. monocytogenes.* Also, 2 out of every 7 poultry samples were positive (111). Farber *et al.*(1) analysed 110 samples of vegetables and 14 samples of raw milk, at the retail level in Ottawa, and all were free from *L. monocytogenes.*

In contrast their survey also found 56.3% of the chicken legs and 86.4% of ground meats sampled contained the organism. Five hundred and thirty ice-cream samples were obtained from manufacturers across Canada and analysed and only 2 were positive for *L. monocytogenes.* In a survey of residential refrigerators for the presence of *L. monocytogenes* in Texas, researchers swabbed 195 refrigerator surfaces and *L. monocytogenes* was not recovered (66).

The Danish Dairy Board emphasized the need to take precautions to protect products from contamination in a booklet in October, 1987, How to avoid listeria in cheese(2). In the Netherlands, 17 food factories were assessed for the occurrence of *Listeria* species. *Listeria* was found in drains, floors, and food contact surfaces (28). In a survey of 35 Dutch household kitchens, 20% were found to be positive for *Listeria* (28). A more recent study in 1992 by Varabioff (114) of 342 samples of retail products from delicatessen and butcher shops in Australia, found that *L. monocytogenes* was recovered from 45 (13.2%) of the samples.

Data on the incidence of listeriosis in Australia has been reported only since 1991. The number of cases since then have been recorded at a rate of about 40 per year (38). In a total of 606 food samples in Singapore investigated for listeria contamination, 2.3% tested

positive for *L. monocytogenes*(89).

These surveys again indicate that *L. monocytogenes* is ubiquitous and present in the food plant environment as well as our food supply (41). Such isolation of *L. monocytogenes* to determine the extent to which this organism is present in various kinds of foods has led to the development of various isolation and genetic techniques.

2.2 Isolation and genetic techniques

The recent food outbreaks discussed earlier have emphasized the need for a highly selective medium to detect *L. monocytogenes*. Techniques to isolate *L. monocytogenes* from foods by direct plating have been uniformly unsuccessful, and therefore successful isolations uses an enrichment in 1 or 2 stages before isolation on a solid media (82). Procedures used to identify *Listeria monocytogenes* include: cold enrichment (54, 55), direct plating (51), a selective enrichment procedure (125), FDA procedure and USDA procedure (55, 125), HPB procedure (39), DNA hybridization (91, 108) and ELISA (108, 112) and fluorescent antibody techniques employing flow cytometry (125). A lot of work has been done comparing the performance of these officially recognized broths, agar and methodology.

The procedure for isolating *L. monocytogenes* used by the FDA was developed by Lovett, Francis and Hunt (54). Twenty-five gram samples are added to 225 mL of *Listeria* enrichment broth (LEB), then homogenized in a stomacher and incubated at 30°C. After 48 h of incubation, the culture is surface plated directly onto modified McBride agar (MMA).

This procedure was modified only slightly by Health Protection Branch of Canada by plating the culture on LiCl-phenylethanol-moxalactam (LPM) and also Modified Oxford Medium, followed by confirmation techniques. (39)

The USDA procedure uses LiCl-phenylethanol-moxalactam (LPM) agar or modified Oxford (MOX) agar (55). In comparing the cold enrichment method (CE) to the USDA method, the USDA method was significantly better ($P \leq 0.001$) than the CE method which took 8-9 weeks for confirmation (55).

In a comparison of MOX medium to an enhanced haemolysis agar (EHA), the data showed EHA had advantages for both the isolation and the discrimination between haemolytic and non-haemolytic species of this genus. It therefore constitutes a good pre-confirmation test (29). In a study on artificially inoculated food products, LPM agar was more sensitive than MMA agar for isolation in terms of recovery of the inoculum (60).

The suitability of 10 direct plating media for isolating and enumerating 4 strains of uninjured and injured *Listeria monocytogenes* from meat, specifically ham and oysters, was evaluated. The LPM agar was selected as the most suitable medium, while Dominguez Rodriguez Isolation Agar (DRIA) was more suitable overall for isolation of *Listeria* from oysters (22, 52). More recent detection techniques, such as the ELISA of the Organon Teknika Corporation, were compared to the commercially available GENE-TRAK DNA probe method and both were contrasted with the FDA procedure. The ELISA detected 68% of the positive samples and the DNA probe only 45%, but the FDA method detected 75% of the positive inoculated food samples (56).

Further research by Vanderlinde showed the ELISA kit by TECRA® detected 72 out of 74 positive samples that were detected initially by enrichment and selective plating. The ELISA kit gave only 2 false negatives and 2 false positives in the study (112).

Enzyme immunoassays and hybridization assays using DNA probes discussed above are not species specific for *Listeria monocytogenes*, but are *Listeria* genus specific. Species specific DNA probes are being tested in food but are presently not readily commercially available (85). API®Listeria and Micro®ID are one-step identification tests which differentiate *Listeria monocytogenes* from other *Listeria* species. Blood Agar is used to distinguish *L. monocytogenes* and *L. ivanovii* from other species by ß-Hemolysis and these strips use sugar fermentations to distinguish between these 2 species.

Flamm (45) cloned a 5.2 kb fragment of the chromosomal DNA of *Listeria monocytogenes* serotype 1/2a, thought to contain the gene coding for beta-haemolysin, into pBR325, which was then subcloned into pUC8; this construction was designated pRF106. The fragment was used to detect *Listeria monocytogenes* in a Southern blot. However, Kim (71) found that the pRF106 fragment cross-hybridized to an isolate of *L. seeligeri*. Kim *et al.* subcloned pRF106 fragment into a M13 bacteriophage vector and sequenced it. The sequence information was used to develop a *Listeria monocytogenes* specific nonisotopic colony hybridization assay to confirm the presence of *L. monocytogenes* on LPM agar plates (72). The probe did not react with other *Listeria* species and the probe used in the colony blot assay had a sensitivity of 100% and specificity of 97%.

Peterkin *et al.* (94) developed a probe specific for *Listeria monocytogenes* from a beta

haemolytic recombinant clone from a *L. monocytogenes* library. The probe was labelled with horseradish peroxidase and used in a direct colony hybridization method on a grid membrane filter. This nonisotopic probe was contrasted with other ^{32}P-labelled DNA probes for detection of *Listeria monocytogenes* and the results indicated no significant difference between the accepted colony hybridization technique using colony blot and the chromogen-labelled DNA probe (91, 94).

Slade and Thompson at the University of Guelph used 9% denaturing polyacrylamide gels to generate low molecular weight RNA (5S rRNA and tRNAs) profiles of several Gram positive species. The profiles of the 5 *Listeria* spp. tested were identical and different from other Gram positive species tested (109). Therefore, their method is a simple and reproducible method for detection of species.

An attractive alternative tool for the detection of *Listeria monocytogenes* is PCR, since it is highly sensitive and would eliminate the need for enrichment culturing. Several researchers took this route. Furrer *et al.* (47) used PCR to amplify 2 specific DNA fragments of the α and β-haemolysin genes. PCR analysis was used to identify suspected isolates from food, identified by conventional isolation methods as *Listeria monocytogenes*. Subsequent serotyping showed perfect agreement with the PCR results (46).

Wernars *et al.* (120) used parts of the *L. monocytogenes Dth18*-gene and different sets of oligonucleotide primers with chromosomal DNA as a template for the detection of *Listeria monocytogenes* by PCR in soft cheeses. They found amplification strongly inhibited by the extracts from soft cheeses containing the *L. monocytogenes* DNA, but this could be

reduced by purification of the DNA. Still their results were variable and required high levels of cfu's for detection (120).

Datta *et al.* (88) cloned the listeriolysin O gene and developed a gene probe labelled with ^{32}P, which was specific for *Listeria monocytogenes*. Later Dener (34) used PCR to amplify the listeriolysin O gene in *Listeria monocytogenes* as a species-specific detection method.

The methodology for the isolation and detection of *L. monocytogenes* is constantly being researched and assessed with an abundance of new information being published. Research for a successful rapid method with consistent results for the isolation of *L. monocytogenes* from food and environment is ongoing as conventional methods are tedious and are variable in their results (85).

Proctor et al. (99) applied pulse-field gel electrophoresis to link sporadic cases of invasive listeriosis with recalled chocolate milk from Wisconsin in 1994. *L. monocytogenes* isolates from 4 hospitalized patients and an environmental dairy sample resulted in the same *Asc* I restriction endonuclease digestion profile that correlated to that of the chocolate milk isolate. The US Department of Agriculture promoted regulatory and industrial responses to the concerns about listeriosis in the USA and there have been no outbreaks of foodborne listeriosis reported in the USA during the last nine years (74).

2.3 *L. monocytogenes* in vegetable products

Many different vegetables have been analysed for the presence of *L. monocytogenes* (44, 57, 96, 107, 113, 124) with various results. Petran *et al.* (96) surveyed the outer portions of fresh beets, broccoli, cabbage, carrots, cauliflower, corn, head lettuce, leaf lettuce, mushrooms and potatoes and determined that no *L. monocytogenes* was detectable in any of these samples. In contrast Heisick *et al.*(57) sampled broccoli, cabbage, carrots, cauliflower, cucumbers, lettuce, mushrooms, potatoes, radishes and tomatoes and *Listeria* spp was isolated from 6 of the 10 types of produce tested. Only potatoes (25.8% positive) and radishes (30.3% positive) showed significant amounts of *L. monocytogenes* contamination.

A study of the incidence of *L. monocytogenes* in foods available in Taiwan reported 12.2% of all the vegetables sampled were positive for *L. monocytogenes* (124). Recent work by Velani and Roberts (115) revealed 8 of 42 mixed salad samples were positive for *L. monocytogenes* whereas in the samples of individual salad ingredients only 2 of the 108 analysed were positive. This implies that further contamination could occur in the salad preparation or the processing plant.

Bendig and Strangeways (5) report a case of hospital-acquired *L. monocytogenes* which prompted them to examine samples of hospital food for contamination with *Listeria* species. Particular attention was paid to dairy foods, however, only the lettuce yielded growth of *Listeria*. Twelve further samples were examined and 2 were positive and all

samples were from washed lettuce, ready to be consumed by patients. One yielded *L. monocytogenes* serotype 1/2a.

Steinbruegge *et al.* (110) researched the fate of *L. monocytogenes* on ready-to-serve lettuce and serotype 1 was isolated from some uninoculated samples indicating that the organism was naturally present on some of the lettuce heads purchased from retail outlets. Possible sources of contamination of vegetables include soil, water, animal manure, decaying vegetation and effluent from sewage treatment plants(101).

Cabbage and cabbage juice were found to support growth of *L. monocytogenes* at 5°C (10). Although the organism does not grow well it survived for long periods in cabbage juice at 4°C (27). Sizmur and Walker (107) reported growth of *L. monocytogenes* in various salads left at 4°C for 4 d, indicating that the organism can survive and multiply during storage in contaminated refrigerated prepared salads.

An anti-*Listeria* effect has been reported by Nguyen-the and Lund (90) with raw carrot slices stored at 8°C, but the effect was heat-labile and further processing such as blending destroyed the antilisterial activity. Amounts of carrot juice as low as 1% in tryptic phosphate broth substantially reduced *L. monocytogenes* growth after only a 24 hour period (8), but the anti-*Listeria* effect was eliminated when the carrots were cooked. The growth of *L. monocytogenes* was also found to be inhibited by certain Chinese medicinal plant extracts (24). The plants suppressed growth of *L. monocytogenes* Scott A in cabbage juice but this inhibition was eliminated with the addition of protein, suggesting protein in various food systems would interfere with antimicrobial effects.

Vegetable crops used in salads and coleslaw are inevitably contaminated with soil and the presence of *L. monocytogenes* is unavoidable especially if animal manure is applied (113, 118, 119), as in the outbreak of listeriosis associated with coleslaw in Nova Scotia. An association between the consumption of lettuce and other raw vegetables and listeriosis among patients in hospital was investigated by Ho *et al.* (61), who linked sporadic listeriosis in the community with salads. The ability of *L. monocytogenes* to grow in cabbage at refrigeration temperatures has been well documented; growth has subsequently been demonstrated on asparagus, broccoli and cauliflower (7).

2.4 Modified atmosphere packaging of salads

Research in low-oxygen-atmosphere storage of perishable fruits and vegetables dates back more than 150 years (65). Modified atmosphere packaging (MAP) of foods enables substantial extension in shelf life of foods. In MAP, a food product is packaged in an atmosphere which has been modified so that its composition is something other than normal air (58). This contrasts with controlled atmosphere packaging (CAP) in which the food is continually exposed to a defined mixture of gases in a storage chamber. An example is CAP storage of apples in CO_2, which retards respiration rates in order to delay senescence. MAP foods are extended shelf life refrigerated products which were developed to meet a growing consumer demand for fresh, high quality convenience foods (40).

The gases normally used for MAP include carbon dioxide (CO_2), oxygen (O_2) and

nitrogen (N_2) (15, 33, 64). The most important gas for inhibiting bacterial growth is CO_2 (62, 64). However, modified atmosphere conditions can inhibit microorganisms that might warn consumers of spoilage while either allowing or enabling the growth of a pathogen (62), like *L. monocytogenes*, which has the ability to multiply at refrigeration temperatures.

Growth of *L. monocytogenes* Scott A inoculated into shredded cabbage was one hundred fold higher in normal air than in modified (70% carbon dioxide and 30% nitrogen) atmosphere, at 25°C. However, after day six counts decreased to undetectable levels. In contrast, there was a less marked initial increase and a more rapid decline in counts in cabbage stored in the modified atmosphere, (70). Different mixed salads stored at 4°C for 4 d supported the growth of *L. monocytogenes* indicating the organism can survive and multiply during refrigerated storage as prepared salads (107).

Further studies by Beuchat and Brackett (9) on the survival and growth of *L. monocytogenes* on lettuce in modified atmosphere suggested that *L. monocytogenes* was inhibited by the competitive activities of naturally occurring psychrotrophs. Modified atmosphere packaging, using specific pack film gas permeabilities, package atmospheres and package dimensions, has been reported to extend the shelf life of vegetables (14, 20).

Is the risk of listeriosis likely to be increased by the use of extended storage at low temperature, compounded by controlled atmosphere packaging, which extends the storage by maintaining an acceptable appearance ?

MATERIALS AND METHODS

PHASE I

3.1 Experimental design

This experiment was designed to examine the growth of *L. monocytogenes* Scott A in packaged coleslaw. A 3x2x2x10 factorial experiment was replicated 3 times to test the following experiment treatments in all combinations for a total of 360 experimental units:

inoculum level (10^4/g vs 10^6/g vs none)

packaging type (ambient air vs modified atmosphere)

storage temperature (3°C vs 8°C)

storage time (1- 10 d)

A replicated split-split-plot statistical design was used, where the inoculum level (x3) and packaging method type (x2) formed the main blocks. The growth temperatures (x2) and storage time (x10) became the split-split-plot treatment. The design layout is shown in Figure 2. Experimental constraints of the design were as follows: each replication used different fresh ingredients provided by the same supplier but there was no control over the variety of cabbage or how it was grown.

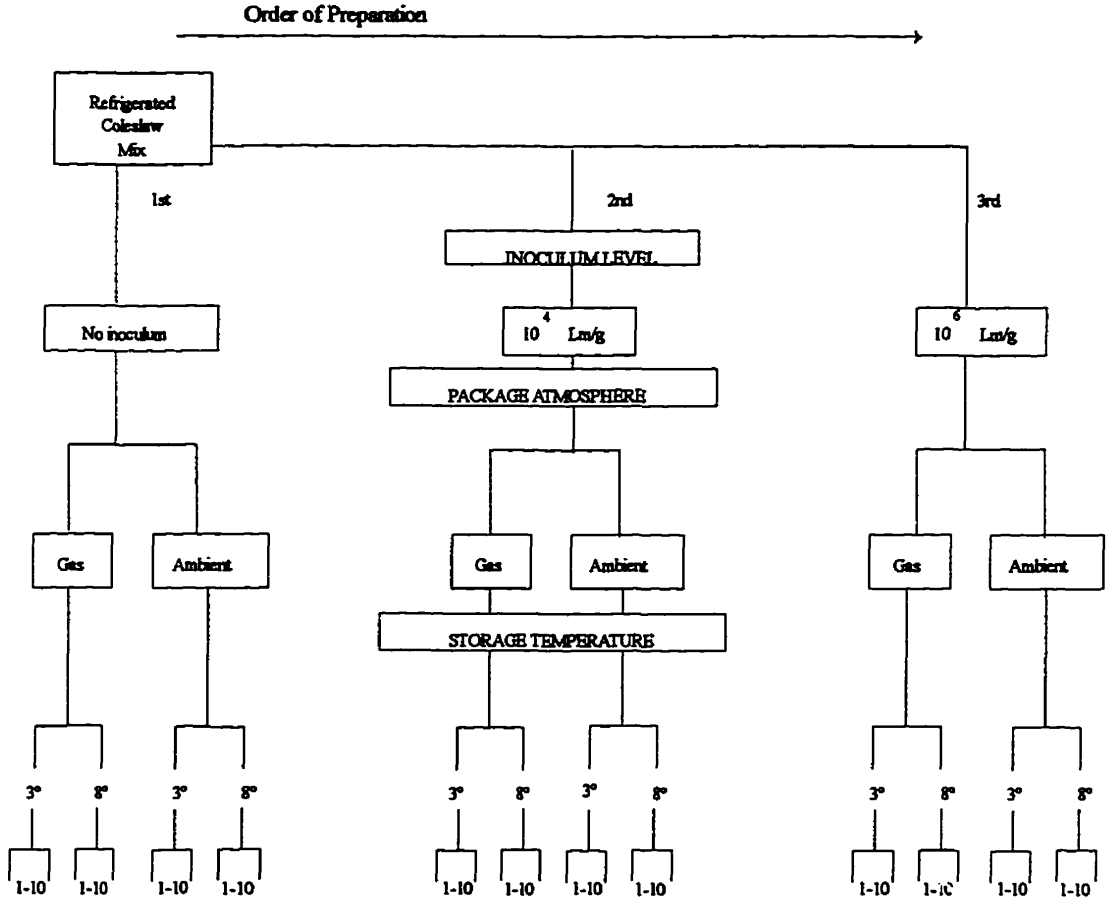

Figure 2. Experimental Design Structure

The prepared coleslaw was inoculated in sequence from none to 10^6/g for each replication (Figure 2), to eliminate cross contamination of inoculated treatments. The same order was used in sealing the packages, again to prevent cross contamination. The sealed packages, for each treatment, were then divided into 2 groups and assigned random placements within the incubator chambers at the 2 temperatures.

Treatment and storage effects were analysed using an analysis of variance (ANOVA) and a polynomial regression analysis was conducted on each attribute. Initially bacterial growth was slow, followed by exponential growth. Therefore to prevent the results from being skewed by the nonsymmetric distribution, all data were transformed to logarithms ($\log_{10}$) prior to statistical analysis. All data were then analysed statistically using Genstat 5 program procedures (80).

3.2 Preparation of coleslaw

The coleslaw was prepared to resemble the commercial product. Twelve packages of commercial coleslaw product were purchased and the pieces weighed and measured to determine the average ratio of carrot to cabbage pieces and the average size of the shredded pieces. A ratio of 7 g of carrot to 100 g of cabbage and the average size for carrot pieces (length 25 mm x 5 mm width x 2 mm thick) and cabbage pieces (70 mm length x 5 mm width x 2 mm thick) was established as typical for commercial coleslaw.

Cabbage and carrot were obtained from a local food broker and rinsed in cold water

to remove any soil contaminants. All equipment, knives, cutting boards and storage containers were sanitized in chlorinated water (200 ppm), and rinsed with sterile distilled water to limit contamination. The cabbage outer leaves were trimmed and heads quartered, and the carrots peeled and shredded using a commercial vegetable processor, model H-600 (Hobart Manufacturing, Don Mills, ON), fitted with a slicer attachment with a 3 mm blade clearance for cabbage and then a #1 grater attachment for carrots. The processed cabbage and carrot were mixed to produce coleslaw with the required 7% w/w carrot to cabbage ratio.

3.3 Preparation of inoculum

The strain of *Listeria monocytogenes* (Scott A, a human isolate from the 1983 Massachusetts outbreak) was obtained from the Bureau of Microbial Hazards, Health Canada, Ottawa. The strain was resuscitated in tryptic phosphate broth (TPB) (Difco Laboratories, Detroit, MI) and streaked on tryptic soya agar (TSA)(Difco Laboratories, Detroit, MI) for purity confirmation. The pure strain was then cultured by the method of Beuchat *et al.* (10) in TPB at 26°C in a Blue M Shaker Incubator model MW1130A-1 (Blue M, Blue Island, IL) for 12 h.

The following method was used to construct a standard growth curve for *L. monocytogenes*. One mL of actively growing culture was used to inoculate 250 mL of TPB. Samples were aseptically removed at 1 h intervals for 9 h. A 4 mL sample of inoculum suspension was immediately transferred to a glass cuvette with a 1 cm light path and the

absorbance of the tube read at 600 nm against a blank of sterile media using a Hitachi UV-VIS Spectrophotometer model 139 (Perkin-Elmer, Tokyo, Japan). The remaining 1 mL sample was used to prepare a dilution series to inoculate duplicate plates of TSA using a Spiral Plater, model CU (Spiral Systems Inc., Cincinnati, OH).

The above method was repeated twice and the average *L. monocytogenes* counts and optical density readings were used to construct a standard growth curve (Figure 3) for estimation of cell numbers in the preparation of inoculum for coleslaw.

The inoculum was prepared for the coleslaw in the following manner. The inoculum was cultured for approximately 6 h and then optical density readings were taken until the cells had reached approximately 10^8 cfu/mL according to Figure 3. This culture was used to prepare several 3 L suspensions by dilution of the inoculum with sterile distilled water to yield a population of 10^6 cfu/mL and subsequently a lower population level of 10^4 cfu/mL. All suspensions were confirmed by plate counts on TSA. A fresh inoculum was prepared in the same manner for each replication of the experiment.

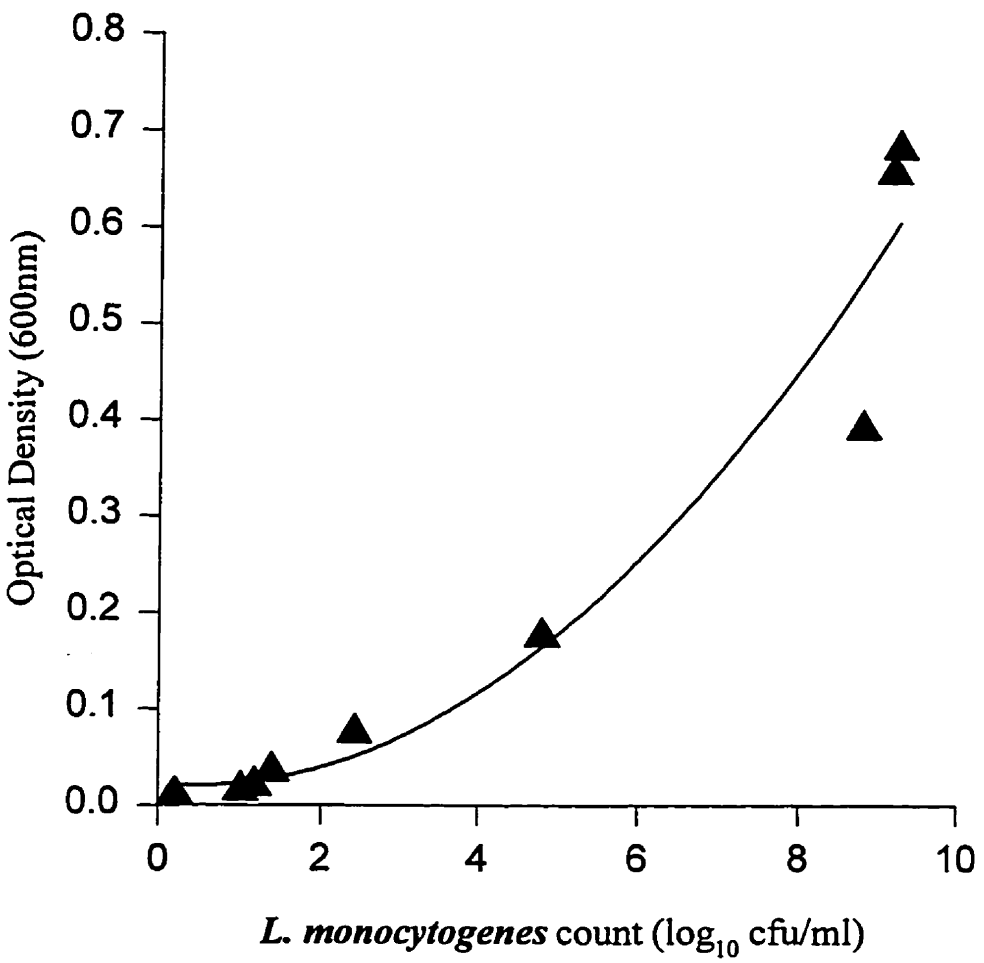

Figure 3. Standard growth curve of *L. monocytogenes* in TPB at 25°C.

3.4 Inoculation of coleslaw with *L. monocytogenes*

The procedure for inoculation of the coleslaw was a modification of Beuchat and Brackett (9). Two kg of coleslaw were placed in an autoclaved round stainless steel colander made from a 10 litre stainless steel mixing bowl (dimensions: 27.0 cm wide and 11.0 cm in depth) with concentric 5 mm holes punched into the bottom and up $^2/_3$ of the side. Each 3 L inoculum suspension was poured into an autoclaved Nalgene® basin (Fisher Scientific, Mississauga, ON) and placed in a Bio Gard Hood, model V40-112 (Baker Co., Sandford, ME). The coleslaw held in the colander was submerged in the inoculum suspension for 30 seconds. It was then removed to a stand and allowed to drain until no liquid dripped from the colander (*ca.* 5 min). The control coleslaw (referred to as none inoculated) was submerged in sterile distilled water and then treated in the same manner. Each batch was thoroughly mixed in preparation for packaging.

3.5 Packaging procedure

Uninoculated control (none) and inoculated (10^4 and 10^6) coleslaw batches were packaged in 200 g quantities in 25 μm low-barrier polyethylene bags (23 cm long x 12.5 cm wide) (Farnell Packaging, Halifax, NS), which are the standard in the local industry. Each packaged inoculum treatment of coleslaw was subdivided into 2 lots (refer to package

atmosphere Figure 2). The first lot was heat sealed directly using a Fresh Vac packaging unit, model A-300, (CVP Systems Inc., Downers Grove, IL) under ambient (normal atmosphere) air. The second lot of packages was evacuated and then flushed with a modified gas mixture before sealing.

The first lots, sealed directly, contained "ambient" or normal air, which is representative of commercial practices. The normal composition of air is O_2 = 21%, CO_2= 0.03% and N_2 = 77% (30), whereas, the modified gas mixture used to gas flush was a 5% O_2 and 5% CO_2 and 90% N_2 gas blend (Atlantic Oxygen Supply, Halifax, NS). The Fresh Vac packaging unit filled each package with a modified gas mixture by a double cycle of evacuation, followed by gas flushing on a snorkel-type packaging attachment, followed by a heat seal. This gas modification was recommended for cabbage and lettuce from studies that examined various modified atmosphere packaging regimes (3,4,15,58). It is important to select a proper modified atmosphere gas mixture for respiring tissue, like coleslaw, so that the package does not become anaerobic and develop abnormal odours during storage. To prevent anaerobiosis, O_2 levels should be maintained at >2% by using a package that is highly permeable allowing sufficient gas transmission to satisfy aerobic metabolic rates within the plant tissues.

3.6 Storage of packages

The packages of coleslaw for each combination of inoculum level and atmosphere

type were subdivided equally between 2 temperatures (3 or 8 ±1°C). Packages were positioned randomly within 2 Isotemp Incubators, 200 series, Model 230D (Fisher Scientific, Mississauga, ON) set at 3°C and 8°C respectively within a controlled 0°C storage room. Storage was maintained for 10 d with 1 package for each treatment being withdrawn daily for microbiological and gas composition analysis.

3.7 Sensory observations

One coleslaw package for each treatment was removed from storage each day for the 10 day storage period. Prior to analysis the general condition of the package, including swelling and discoloration, was recorded in comparison to a fresh commercial package. The presence of any off-odours were noted upon opening packages for microbiological analysis. These sensory observations were made by only one person and hence were subjective and therefore unsuitable for statistical assessment.

3.8 Atmosphere Analysis

Once sensory observations were noted each package was subjected to gas analysis. Each package had a 1" plasticized, rip-resistant tape patch applied to the outer film. Headspace gas samples were taken by piercing the patch with a thin, 22-gauge, sterile

hypodermic syringe (Fisher Scientific, Mississauga, ON) attached to a gas-tight sampling tygon tube. The tube was connected to a semi-automated 2 mL gas sampling port on a Gow Mac GC (gas chromatography), series 580 (Gow-Mac Instruments Co.,Bridgewater, NJ).

The GC was fitted with a thermal conductivity detector set at 220 mA and used a 2 m x ¼" outer diameter concentric CTR1 column packed with Porapak blend (outer column) surrounding a molecular sieve 5A (80-100 mesh) (inner column) (Alltech, Deerfield, IL). Helium was used as a carrier gas maintained at a flow rate of 15 mL min^{-1}. Temperature conditions settings were: column at 40°C; injector at 70°C; and detector at 50°C. The concentrations of O_2, CO_2 and N_2 in gas sample was calculated from sample peak areas at predetermined retention times using a Spectra-Physics integrator, model SP4290, (Spectra-Physiscs, San José, CA). The GC was calibrated using a standardized reference gas mixture of 5% O_2, 5% CO_2 and 90% N_2 (Atlantic Oxygen Supply, Halifax, NS).

3.9 Microbiological analysis

3.9.1 *L. monocytogenes* count

Following gas sampling, coleslaw was aseptically withdrawn from the package, from 5 random areas (*ca.* 5 g) , for a total composite sample of 25-30 g. The sample was weighed into a sterile sample bag held in a tared beaker on a top-loading balance and combined with 0.1% (w/v) sterile peptone (Difco Laboratories, Detroit, MI) to a 1 in 10 dilution. Each sample was pummelled with a Colworth Stomacher, model 400 (A J Steward, London, UK)

for 2 minutes. Dilutions, 10^4 and 10^6, were sequentially prepared in 0.1% peptone and plated on Oxoid Oxford isolation agar (Unipath Inc., Nepean, ON) using a Spiral Plater, model CU (Spiral Systems Inc.,Cincinnati, OH). Plates were incubated for 48 hours at 30°C and read using a Spiral Manual Counter, model MV (Spiral Systems, Cincinnati, OH). Typical *L. monocytogenes* colonies (circular, smooth, metallic black, and convex in appearance) were enumerated.

Limited identification of randomly selected *L. monocytogenes* colonies was conducted using the confirmation method described by Golden *et al.* (52). This included examination of cellular morphology and tumbling motility in wet mounts, catalase production, oxidase reaction, and umbrella-shaped growth in motility test medium. Further confirmation was done by assessment of haemolytic activity on blood agar base (Difco Laboratories, Detroit, MI) containing 5% horse blood (Woodlyn Laboratories, Guelph, ON), and sugar fermentation reactions in 5 mL of sterile purple broth base (Difco Laboratories, Detroit, MI) tubes with filter sterilized 0.5% (w/v) rhamnose or xylose (Sigma Chemical Co., St. Louis, MO) carbohydrate added.

3.9.2 Total aerobic microbial plate counts

The dilutions, 10^4 and 10^6, were also spiral plated on TSA (Difco Laboratories, Detroit, MI). Plates were incubated aerobically at 35°C for 48 h and the cfu/g of coleslaw was calculated.

3.10 Assessment of micro flora

Two colonies were selected from each 10^4 aerobic TSA plate per treatment on day 1 and transferred two consecutive times to TSA for purification. The colonies were selected based on dissimilar characteristics from *L. monocytogenes* colonies and the quantity present. Colony morphology and presence of chromogenesis was recorded. The isolates were further examined using Gram stain, catalase reaction, oxidase (50), and API 20E strips (API Laboratory Products Ltd., St. Laurent, PQ) were used for identification.

PHASE II

3.11 Experimental design

A 96 x 2 factorial experiment was conducted and replicated 6 times, using a 96 well Biolog® GP MicroPlate (Biolog Inc., Hayward, CA) containing a preselected panel of 95 different carbon source wells and one control well, to determine the ability of *L. monocytogenes* to utilize or oxidize these carbon sources at 8 and 30°C. (See Appendix 1 for description of carbon sources.) Experimental design layout is shown in Figure 4.

3.11.1 Statistical analysis

The effect of temperature on the growth of *L. monocytogenes* in carbon sources was analysed using non-linear regression equations in Table Curve® 2D, V.3 (Jandel Scientific Inc, San José, CA). The procedure UNIVARIATE in SAS (Statistical Analysis Software, Carey, NC) was used to test for normality of the data. The data were determined to be non-parametric. Therefore a non-parametric test, the Sign Test, was used to test for differences

$$z = \frac{\overline{p} - p_o}{\sqrt{\frac{p_o (1 - p_o)}{n}}}$$

in means between 8 and 30°C slopes (Dr. Farrell; personal communication).

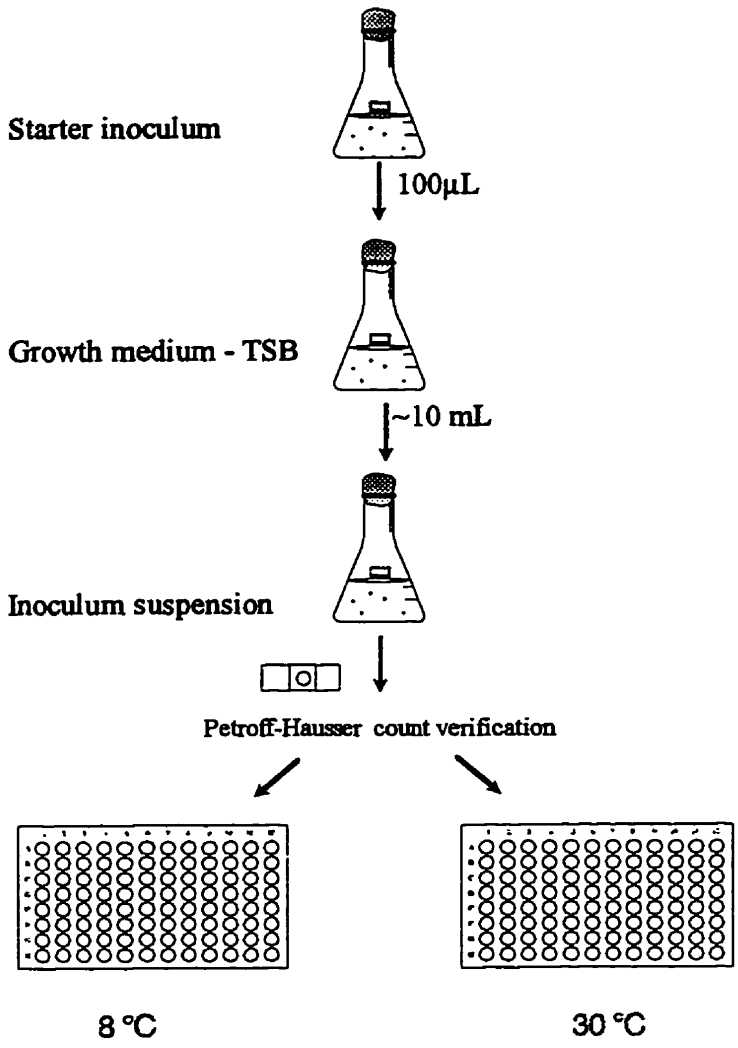

Figure 4. Experimental design layout for Phase II. Represented is 1 of 6 replications.

The " p_o " is the number of positive sample differences, " p " = 0.5 and " n " is the number of wells.

3.12 Preparation and inoculation of microplate

L. monocytogenes Scott A was cultured overnight in 200 mL of tryptic soy broth (TSB) in an environmentally controlled shaker at 35°C. A 100 µL aliquot of the overnight culture was used to inoculate 6 x 250 mL flasks containing 200 mL of TSB medium. All flasks were incubated for 18 hours at 35°C with one flask of inoculum used as an inoculum source for each replicate.

The inoculum suspension was prepared by diluting approximately 10 mL of the 18 h culture of *L. monocytogenes* with approximately 40 mL of 0.85% w/v NaCl (Difco Laboratories, Detroit, MI) sterile saline. The inoculum density was adjusted to between 35-42% T, the acceptable turbidity range guideline given by Biolog® for Gram positive identification. The suspension was then checked with a Petroff-Hausser Counting Chamber (C.A.H.& Son, Philadelphia, PA) to verify that the density was approximately 10^8 cells/mL. Adjustments were made by the addition of more saline or inoculum and vortexed to form a homogeneous cell suspension.

Each microplate was appropriately labelled with the replicate number and start time. Sterile saline, 0.85% w/v NaCl, pH 6, and the microplates were prewarmed to 30°C. All supplies needed for the inoculation of the plates and disposal were placed in the biosafety

cabinet and equilibrated to room temperature. The Biolog® turbidimeter was calibrated with Biolog® turbidity standards and a tube of uninoculated 0.85% w/v saline was used to calibrate the 100% transmittance. The standardized inoculum suspension was poured into a multichannel pipette reservoir (Fisher Scientific, Mississauga, ON). A 12 channel micropipetter (Costar, Cambridge, MA) was used to pipette the inoculum into the Biolog® microplates. To assure tips were dispensing the correct volume, 150 µL double distilled deionized water was pipetted into a tared weighboat placed on an analytical balance. Aliquots of 150 µL of the suspension were pipetted into each microplate well, assuring no carry over or cross contamination occurred between additions. The same suspension was used for both 8°C and 30°C microplates within each replicate as indicated in figure 4. Microplates were covered with the provided lids and incubated at either 8°C or 30°C. All microplates were stored during incubation in closed containers to prevent evaporation. Two sterile paper towels were soaked with sterile distilled water and placed in the bottom of each container to minimize dehydration of the outer wells of the microplates.

3.13 Microplate data collection

Each plate was analysed with the Biolog® MicroPlate Reader (Biolog Inc., Hayward, CA) following addition of the suspension (time zero) and at various time intervals up to 150 h for plates incubated at 8°C, and up to 90 h for plates incubated at 30°C. Each plate reading was saved in two different formats, 1) raw optical density readings; 2) Biolog's

conversion of the data to positive ("+"), negative ("-") and variable ("v") well designations. These designations are based upon a percent change which was calculated from the raw optical densities using the A1 control well as the " zero". An optical density reading ≥ 40% change indicated a positive designation. Wells with readings close to the control (<10%) were designated as negative. The variable designation represented values that are borderline to the 40% threshold. The positive, negative and variable well designations after 24 h incubation for all 30°C microplates were averaged and the "+" and "-" pattern codes were converted manually to a simplified octal code number, called a bio-number. This bio-number is a 32 digit number calculated by entering the "+" and "-" designations into a template supplied by Biolog®. Each well has a designated "score", either 4, 2 or 1. After entering the "+" or "-" into this template scores in the wells containing a "+" in 3 adjacent wells (i.e. A1 to A3, A4 to A6, etc) were summed into one number. Negatives within the template were scored as zero. This bio-number was then used for comparison to the Biolog® library data base to verify a *Listeria spp.* identification.

RESULTS

Phase I Results

In this experiment the effects of modified atmosphere on *L. monocytogenes'* ability to increase in numbers in the substantial microbial background in coleslaws was studied in a 3 x 2 x 2 x 10 factorial design (see figure 2). The design consisted of those factors and levels detailed in figure 2. The means ($\log_{10}$) were calculated for each factor and analysed by ANOVA and regression to measure the effects of the experimental treatments as well as to display the pattern of survival of the organism . Results of these analysis are given in the Tables II-IV and Figures 5-8. SEM is the standard error of the mean values presented in the tables and indicates the level of significance of each treatment. The "n" is the number of experimental units and "df" is the degrees of freedom for the factor being examined. Regressional analysis models may be described as linear (L) or quadratic (Q).

4.1 Zero time values

Initial *L. monocytogenes* and total bacterial counts in the packages immediately after preparation of the coleslaw were averaged over the 3 repetitions and are shown in Table I. The initial gas percentages in modified atmosphere packages are also recorded in Table I.

Table I. *L. monocytogenes* numbers, total aerobic microbial counts, and atmospheric composition of the modified atmosphere packages immediately after the preparation of packaged coleslaw (time zero).

Source of variation	*L. monocytogenes* (log_{10} cfu/g)	Total bacteria (log_{10} cfu/g)	O_2 (%)	CO_2 (%)
No *Listeria* inoculum	0	5.13 ±0.25*	5 ±0.05	5 ±0.05
10^4 *Listeria*/g coleslaw	3.86 ±0.84	5.60 ±0.45	"	"
10^6 *Listeria*/g coleslaw	5.54 ±0.69	5.98 ±0.55	"	"

* Log of the Mean ± Standard deviation.

4.2 Inoculum level

Table II illustrates that the inoculum level did not have a significant effect on *L. monocytogenes* or total bacterial counts averaged over all the factors. The higher inoculum of 10^6 therefore did not give *L. monocytogenes* a competitive advantage against the normal flora of coleslaw. The "none" inoculated coleslaw was not included in the analysis of the affects of inoculum level and is reported as " zero" in Table II.

The percentages of either CO_2 or O_2 were also not significantly different for the 3 inoculum levels. Thus the metabolic activity of both the *L. monocytogenes* and the normal flora did not alter the gaseous environment beyond that caused by the respiring plant tissue. The overall O_2 level remained at 2-3% in the packages, indicating that the packages remained aerobic throughout the duration of this study.

4.3 Package atmosphere

Table III shows that the elevated CO_2 and O_2 percentage in the modified atmosphere packages compared to ambient air sealed packages did not significantly affect growth of either *L. monocytogenes* or total bacteria counts. There were also no significant differences evident between the CO_2 or O_2 percentages sampled from both treatments.

Table II. The effect of inoculum levels on *L. monocytogenes* numbers and mean bacterial counts and atmospheric composition in packaged coleslaw over all the treatments.

Source of variation	*L. monocytogenes* (log_{10} cfu/g)	Total bacteria (log_{10} cfu/g)	O_2 (%)	CO_2 (%)
No *Listeria* inoculum	0	7.18	2.09	22.12
10^4 *Listeria*/g coleslaw	5.58	7.26	2.91	18.36
10^6 *Listeria*/g coleslaw	6.45	7.33	2.90	18.17
SEM	0.394	0.088	0.291	1.650
Significance (P)	0.256	0.529	0.186	0.294
	(n=120,df=2)	(n=120,df=4)	(n=120,df=4)	(n=120,df=4)

Table III. Effect of initial modified atmosphere package treatment on *L. monocytogenes* numbers, mean total bacterial counts and final atmospheric composition in packaged coleslaw over all the treatments.

Source of variation	*L. monocytogenes* (log_{10} cfu/g)	Total bacteria (log_{10} cfu/g)	O_2 (%)	CO_2 (%)
Ambient atmosphere	6.10	7.18	2.54	18.98
5%O_2/5%CO_2/90%N_2	5.93	7.33	2.70	20.11
SEM	0.099	0.051	0.210	1.074
Significance (P)	0.332	0.168	0.647	0.535
	(n=120,df=2)	(n=180,df=2)	(n=180,df=2)	(n=180,df=2)

4.4 Storage temperature

Storage temperature did significantly affect counts of *L. monocytogenes* and total bacteria ($P<0.001$), shown in Table IV. At 8°C the counts of *L. monocytogenes* and total plate count were over 1 log higher than subsequent counts recorded at 3°C. Consequently, slight temperature abuse during storage can promote increased growth, not only of the natural flora, but more importantly *L. monocytogenes*. The percentage of oxygen was also significantly higher when storage temperature increased. The increase in oxygen was 1.3 percentage points higher at 8°C, therefore O_2 at 8°C is approximately 69% higher than 3°C O_2 levels.

During the ten days of storage, sensory observations were recorded for all treatments, as shown in Table V. All packaged coleslaw showed no visible signs of spoilage until day 6 of storage. At this time, the gas flushed packages inoculated with either 10^4 or 10^6 cfu/g *L. monocytogenes* at 8°C were the first to show visible signs of deterioration, as indicated by swelling of the package. The ambient sealed packages, however, had no obvious defects until day 9 at 8°C storage, at which point the packages had swollen and the coleslaw had developed an off odour and discoloration. Interestingly, ambient packages stored at 3°C did not show any signs of spoilage for the entire storage period, while gas flushed packages, whether inoculated with *L. monocytogenes* or not, started to deteriorate.

Table IV. The effects of storage temperature on *L. monocytogenes*, mean total bacterial counts, and the atmospheric composition of packaged coleslaw over all the treatments.

Source of variation	*L. monocytogenes* (log_{10} cfu/g)	Total bacteria (log_{10} cfu/g)	O_2 (%)	CO_2 (%)
3°C Storage	5.43	6.88	1.96	18.98
8°C Storage	6.60	7.64	3.31	20.12
SEM	0.149	0.054	0.232	0.724
Significance (P)	<0.001	<0.001	<0.001	0.286
	(n=120,df=152)	(n=180,df=228)	(n=180,df=228)	(n=180,df=228)

Table V. Sensory observations of each package of coleslaw for presence of package swelling, odour or colour deterioration.

		Sensory Observations											
		Package Swelling				Colour Deterioration				Product Odour			
Storage	Inoculum	Ambient		Gas Flush		Ambient		Gas Flush		Ambient		Gas Flush	
		3°C	8°C	3°C	8°C	3°C	8°C	3°C	8°C	3°C	8°C	3°C	8°C
1	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
2	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
3	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
4	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
5	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
6	none	-	-	-	-	-	-	-	-	-	-	-	-
	10^4	-	-	-	+	-	-	-	-	-	-	-	-
	10^6	-	-	-	+	-	-	-	-	-	-	-	-
7	none	-	-	-	+	-	-	-	-	-	-	-	-
	10^4	-	-	-	+	-	-	-	-	-	-	-	-
	10^6	-	-	-	+	-	-	-	-	-	-	-	-
8	none	-	-	+	+	-	-	-	+	-	-	-	+
	10^4	-	-	-	+	-	-	-	+	-	-	-	+
	10^6	-	-	+	+	-	-	-	+	-	-	-	+
9	none	-	+	+	+	-	-	+	+	-	+	-	+
	10^4	-	+	+	+	-	-	+	+	-	+	-	+
	10^6	-	+	+	+	-	-	+	+	-	+	-	+
10	none	-	+	+	+	-	+	+	+	-	+	+	+
	10^4	-	+	+	+	-	+	+	+	-	+	+	+
	10^6	-	+	+	+	-	+	+	+	-	+	+	+

4.5 Length of storage time

Mean counts of both *L. monocytogenes* and total bacterial counts, when averaged over all the treatments, increased significantly ($p \leq 0.001$) with storage time. Both counts had increased approximately 2 log cycles by day 6 of storage from the mean counts on day 1. After day 6 the counts peaked and then remained at approximately the same level for the duration of the study. The mean *L. monocytogenes* counts remained approximately 1 log cycle lower than mean total bacteria counts for each of the 10 days.

The oxygen concentration level increased from 1.4% at day 1 to 4.2% at day 5; dropped to 2.1% on day 6; then increased again to 4.3% on day 10. The percentage CO_2 fluctuated from the lowest level of 17.3% to a high of 22.7%.

Table VI. The effect of each day of storage time on *L. monocytogenes*, total bacterial counts and atmospheric composition of the coleslaw package.

Source of variation		*L. monocytogenes* (log_{10} cfu/g)	Total bacteria (log_{10} cfu/g)	O_2 (%)	CO_2 (%)
Storage time	1	4.84	5.95	1.41	17.31
(Days)	2	5.38	6.28	1.30	20.37
	3	5.79	6.73	2.56	18.71
	4	6.20	7.07	2.16	20.04
	5	6.27	7.52	4.23	19.92
	6	6.23	7.75	2.13	20.13
	7	6.20	7.78	2.47	19.97
	8	6.34	7.83	2.58	22.67
	9	6.43	7.76	3.25	17.31
	10	6.51	7.88	4.26	17.86
SEM		0.124	0.089	0.440	1.290
Significance (P)		<0.001 (L,Q)[1]	<0.001 (L,Q)[1]	<0.001 (L)[1]	0.002 (Q)[1]
		(n=24,df=152)	(n=36,df=228)	(n=36,df=228)	(n=36,df=228)

[1] L = Linear; Q = Quadratic

4.6 Interaction between inoculum level and temperature

Figure 5 shows interactions between inoculum level and temperature for all measured responses. There were no significant differences between the inoculum treatments on average counts of *L. monocytogenes* at 3°C or at 8°C. However, the *L. monocytogenes* average count at 3°C for the 10^4 inoculum was significantly different from the average count at 8°C for the10^6 inoculum level. The relevance of this observation is unknown.

The mean counts of total bacteria were not significantly different between inoculum levels at 3°C or at 8°C. The lowest average total counts were obtained at 3°C when the *L. monocytogenes* was at the lower inoculum or absent. The mean total count of coleslaw without an inoculum (none) stored at 3 °C was significantly different from mean total counts of all inoculum levels stored at 8 °C, indicating higher average counts were obtained at the higher temperature.

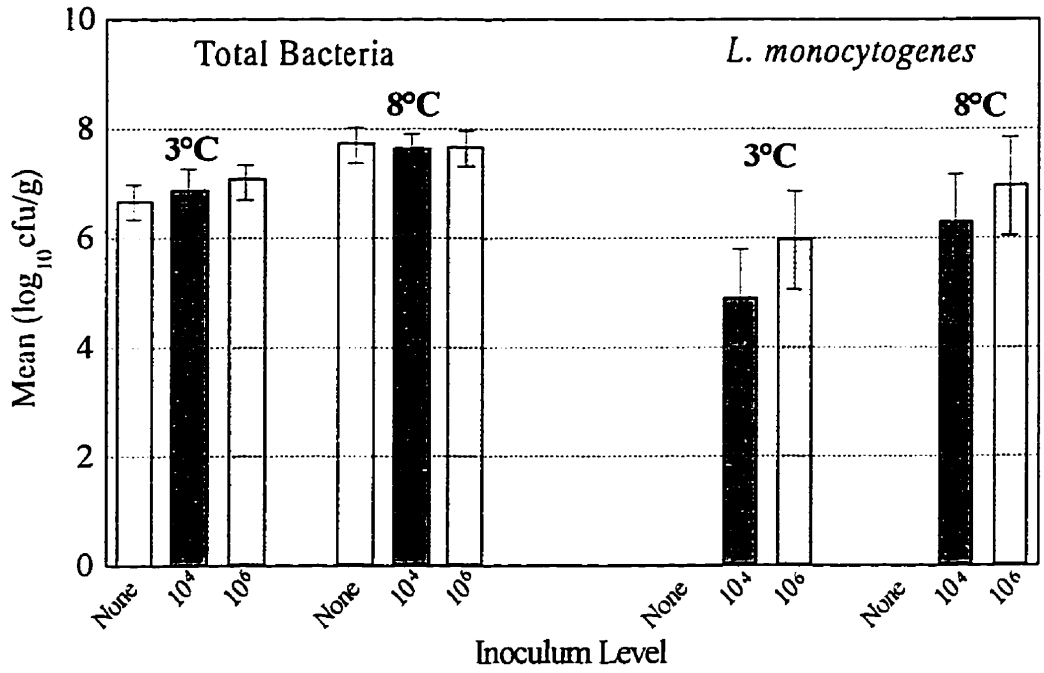

Figure 5. The effects of initial inoculum level and storage temperature on mean number of *L. monocytogenes*/g ($P \leq 0.039$, SEM= 0.300, n=60, df=228) and mean total bacterial counts, cfu/g ($P \leq 0.004$, SEM=0.108, n=60, df=228) in coleslaw. Bars indicate the least significant difference ($LSD_{0.05}$) values.

4.7 Interaction of storage temperature with time

Growth trends during storage for *L. monocytogenes* and total bacterial counts are shown in figure 6. There was no significant difference between the growth rates for *L. monocytogenes* at 3 and 8°C ($p \leq 0.539$), although counts were generally lower at 3°C. In the case of mean total bacterial counts the growth trends slowly converge over time at the two temperatures. The trend may be due to the possible growth of psychrotrophic organisms naturally present on the coleslaw in addition to the *L. monocytogenes* present.

The trends in gas composition in the coleslaw package during storage as influenced by temperature are shown in Figure 7. The mean percentages of O_2 and CO_2 were significant ($p \leq 0.001$) for the interaction of storage temperature with storage time for all measured responses. Percent O_2 increased at 8°C but remained static at 3°C. Under the same conditions, CO_2 decreased at 8°C and increased at 3°C. One gas increased and the other decreased to the point where the percentage of CO_2 at both temperatures crossed over at Day 7. The percentage of CO_2 at 3°C continued to increase and at 8°C decrease (Day 7-10). These changes are more likely related to the metabolic activities of the cabbage cells than to microbial growth. The senescence of the packaged coleslaw at 8°C was more rapid than at 3°C . Reduction of the concentration of both gases resulted in gradual equilibration of the package atmosphere to the surrounding ambient air.

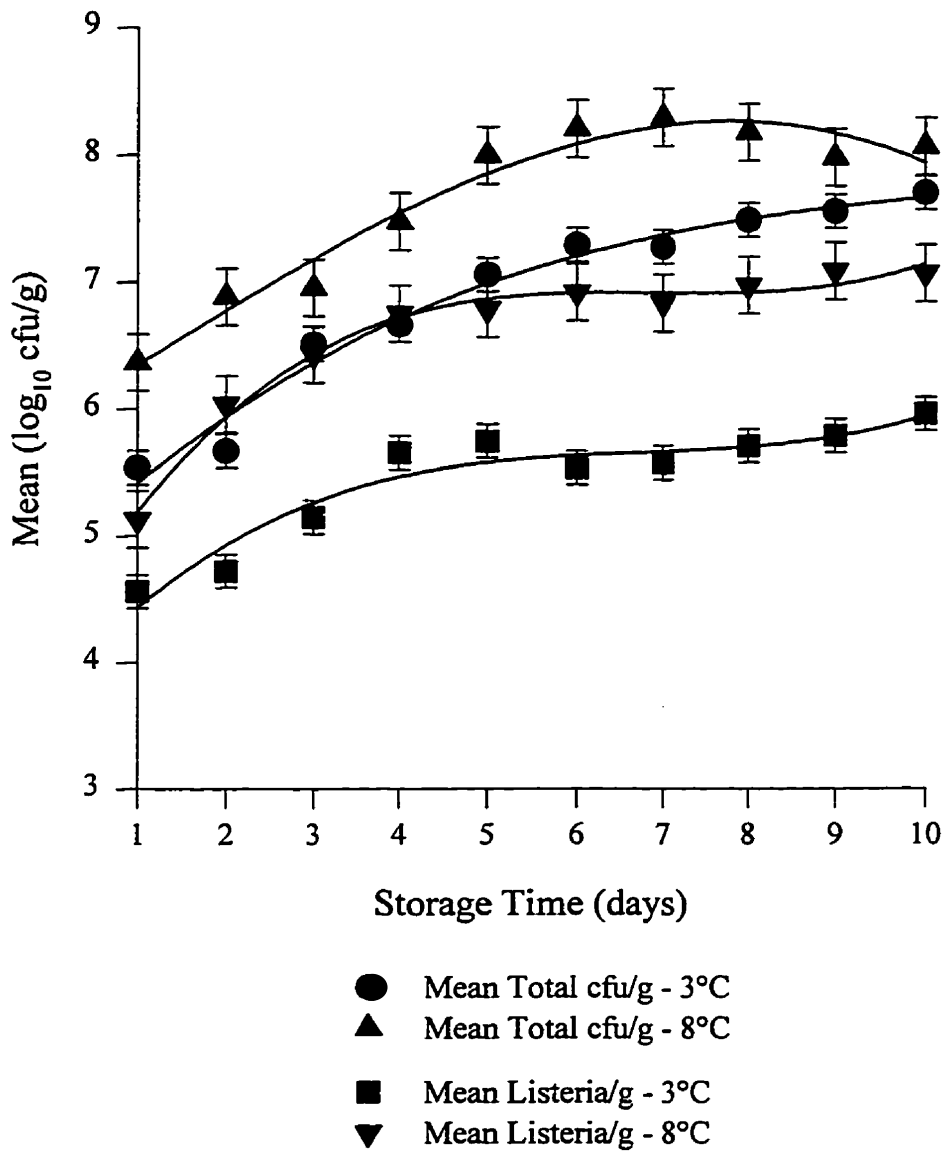

Figure 6. The effects of storage temperature and length of time in storage on mean number of *L. monocytogenes*/g ($p \leq 0.539$, SEM=0.224, n=12, df=152) and mean total bacterial counts, cfu/g ($p \leq 0.012$, SEM=0.132, n=18, df=228) in coleslaw.

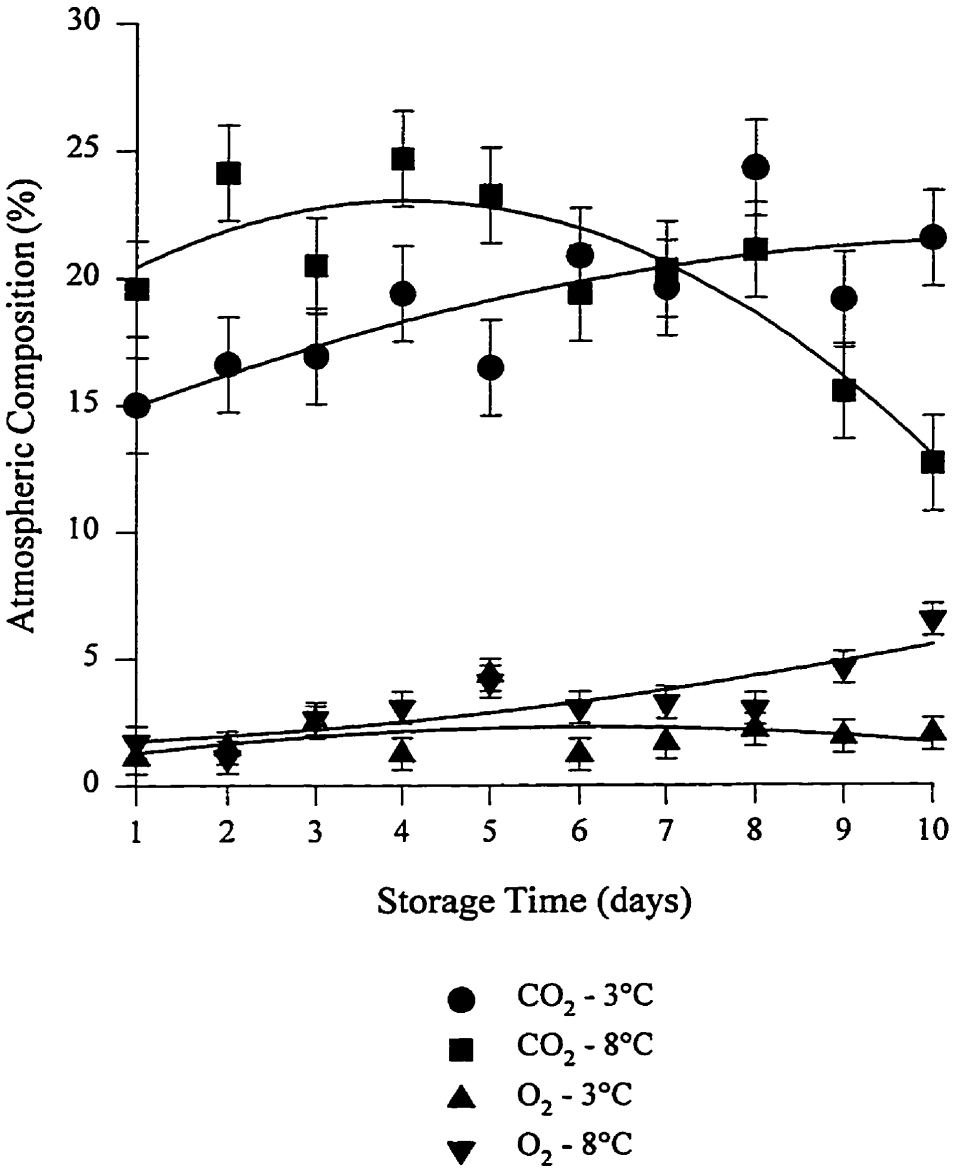

Figure 7. Interaction between storage temperature and the length of storage time on mean percent O_2 ($p \leq 0.001$, SEM=0.635, n=18, df=228) and mean percent CO_2 ($p \leq 0.001$, SEM=1.876, n=18, df=228) content of the package.

Figure 8 depicts the interaction between storage temperature, package atmosphere, and inoculum levels on mean total bacterial counts for all measured responses. The interaction between these factors was not significant except for the coleslaw "none" inoculum stored at 3°C in ambient packages when contrasted to levels of total bacteria at 8°C.

4.8 Assessment of microflora

Table VII lists 10 bacterial isolates identified from the total bacterial isolation media plates incubated on day 1. This analysis of the microflora was conducted to assess the types of bacteria competing with the *L. monocytogenes* inoculum. The predominant micro flora was Gram-negative rods, with *Pseudomonas* and *Serratia* species being the most numerous.

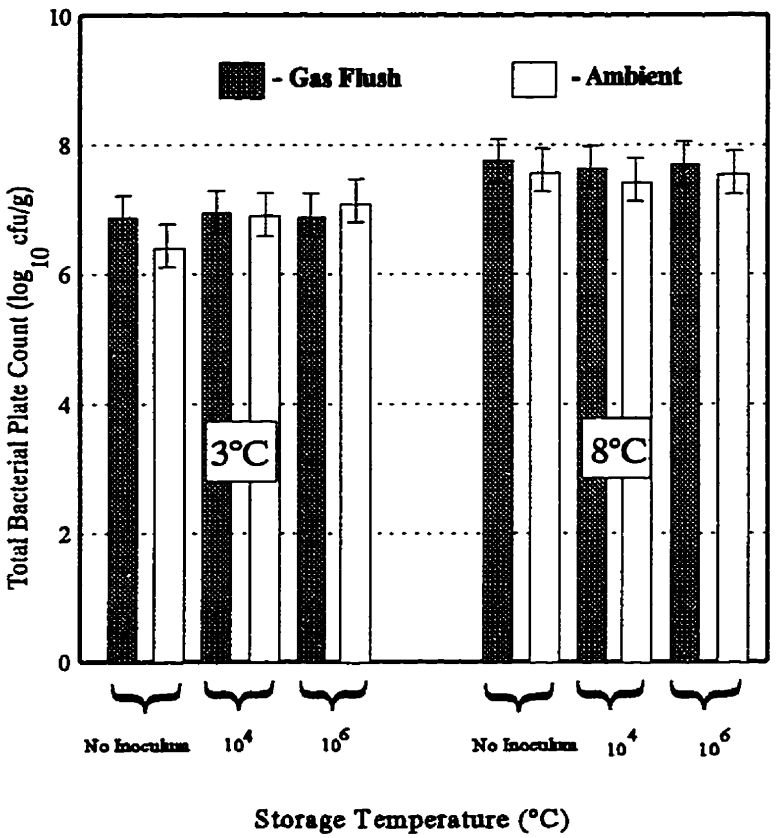

Figure 8. Effects of storage temperature, packaging process, and *L. monocytogenes* inoculum levels on mean bacterial counts (cfu/g) of packaged coleslaw ($p \leq 0.056$, SEM=0.141, n=30, df=2).

Table VII. Results of identification tests on 10 randomly selected bacterial isolates from commercial coleslaw.

Isolate #	Morphology	Gram Stain	API 20E Number	Cat§	Ox*	API 20E Identification
1	rods	G-	2206006	+	+	*Pseudomonas aeringenosa*
2	rods	G-	0006525	+	+	*Pseudomonas pseudomallei*
3	rods	G-	1207727	+	+	Unidentified
4	rods	G-	1007133	+	-	*Enterobacter agglomerans*
5	rods	G-	2203004	+	+	*Pseudomonas fluorescens*
6	rods	G-	2002024	+	+	*Chromobacterium spp.*
7	rods	G-	1207763	+	-	*Serratia plymuthica*
8	cocci	G-	2222042	+	-	Unidentified
9	rods	G-	1207723	+	-	*Serratia ficaria*
10	rods	G-	1007042	+	+	Unidentified

§ - Catalase test
* - Oxidase test

Phase II Results

4.9 Verification of *L. monocytogenes* using Biolog®

The mean optical densities of *L. monocytogenes* cultured at 30°C at 24 h growth were converted to a "bio-number". This number was compared to Biolog® 's library database for identification of unknown microorganisms. Biolog® reported a positive identification for *Listeria spp.*

There were 23 carbon source results in the experiment that differed from the library database environmental identification listing for *L. monocytogenes.* The identification by Biolog® was not conclusive for *L. monocytogenes* but was conclusive for *Listeria spp.*

4.10 The growth of *L. monocytogenes* in a Biolog® MicroPlates at 8 and 30°C

Growth data were used to derive "best fit" curves for each of the 33 carbon sources utilized by *L. monocytogenes* at 8°C and 30°C incubation. Optical density readings were recorded at various intervals over a 150 h time period at 8°C and a 90 h time period at 30°C. These values over time were used to generate the experimental data growth curve using Jandel Scientific® Table Curve™ 2D V.3, a nonlinear regression program, to "best fit" a curve to the data. As growth of *L. monocytogenes* was distinctly different between 8°C and 30°C, each set of data had to be analysed separately.

Growth at 8°C was linear over time, therefore it was analysed using a linear regression equation (y=mx+b). At 30°C, growth followed a hyperbolic pattern; therefore the following equation, y=a+bx/(c+x), was applied to "best fit" the data to a hyperbolic curve. These results are shown in Table VIII.

The two parameters, also given in Table VIII, maximum asymptote and ½-saturation constant, were subsequently used to compare growth on the various carbon sources. Graphs of the analysis for each carbon source are listed in Appendix II.

L. monocytogenes' growth in the majority of the carbon sources exhibited hyperbolic growth at 30°C except for 3 wells. The D-arabitol (well B2), gentiobiose (well B9) and sucrose (well D12) carbon sources resulted in a growth pattern which was better described by a sigmoidal growth equation , y=a+b/(1+exp(-(x-c)/d)). Growth in these wells at 8°C was recorded as positive for the D-arabitol (well B2), gentiobiose (well B9) and negative for sucrose (well D12). Growth responses within these three wells are also shown in Appendix II, but were not included in the overall analysis. Biolog® wells displaying no increase over the initial inoculum optical density were designated as nongrowing and were not submitted to curve fitting. The optical density declined in some wells after attainment of maximal population densities at 30°C.

Table VIII. Line of best fit for results of *L. monocytogenes* growth at 8°C compared to best fit of the curve for growth at 30°C on the same carbon source. (Listed are all the carbon sources that *L. monocytogenes* utilized for growth at these two temperatures. *L. monocytogenes* did not sufficiently utilize the other 62 carbon sources.)

WELL	CARBON SOURCE	GROWTH AT 30°C			GROWTH AT 8°C	
		maximum asymptote	½-saturation constant	slope	y-intercept	slope
A4	dextrin	0.396	0.344	0.022	0.281	-1.867×10^{-4}
A10	N-acetyl-D-glucosamine	2.156	2.062	0.437	0.362	7.813×10^{-4}
A11	N-acetyl-D-mannosamine	2.265	2.109	0.310	0.314	3.445×10^{-4}
A12	amygdalin	0.992	0.844	0.121	0.217	-4.311×10^{-4}
B3	arbutin	1.915	1.849	0.387	0.340	2.229×10^{-3}
B4	cellobiose	1.888	1.756	0.309	0.401	3.086×10^{-3}
B5	D-fructose	1.956	1.858	0.316	0.504	2.828×10^{-3}
B7	D-galactose	0.689	0.609	0.085	0.333	-5.253×10^{-5}
B11	α-D-glucose	2.009	1.903	0.416	0.476	1.714×10^{-3}
C2	lactulose	0.612	0.474	0.039	0.249	-2.486×10^{-4}
C4	maltotriose	0.285	0.183	0.008	0.267	5.538×10^{-5}
C5	D- mannitol	2.097	2.014	0.316	0.237	1.644×10^{-4}
C10	ß-methyl D-galactoside	0.699	0.611	0.019	0.264	-2.887×10^{-4}
D1	ß-methyl D-glucoside	2.359	1.807	0.197	0.276	2.353×10^{-3}
D4	D-psicose	2.179	1.886	0.195	0.468	5.674×10^{-4}
D7	D-ribose	0.657	0.484	0.058	0.364	-2.76×10^{-4}
D8	salicin	2.373	2.219	0.314	0.424	3.393×10^{-3}
E1	D-tagatose	0.831	0.763	0.040	0.305	-2.422×10^{-4}
E2	D-trehalose	1.686	1.612	0.200	0.298	1.649×10^{-3}
E4	xylitol	2.160	0.948	0.095	0.291	1.378×10^{-4}
E5	D-xylose	0.599	0.517	0.071	0.429	-1.407×10^{-4}
F6	methyl pyruvate	1.108	1.070	0.084	0.261	5.018×10^{-4}
G12	glycerol	1.807	0.843	0.066	0.322	9.274×10^{-4}

(Continued from page 56)

WELL	CARBON SOURCE	GROWTH AT 30°C			GROWTH AT 8°C	
		maximum asymptote	½-saturation constant	slope	y-intercept	slope
H1	adenosine	0.874	0.815	0.093	0.237	-2.630×10^{-4}
H2	2'-deoxy adenosine	0.593	0.325	0.087	0.265	-2.428×10^{-4}
H3	inosine	0.760	0.612	0.097	0.235	-3.843×10^{-5}
H5	uridine	0.982	0.762	0.203	0.226	2.458×10^{-4}
H6	adenosine-5'-monophosphate	0.526	0.261	0.045	0.227	1.146×10^{-5}
H8	uridine-5'-monophosphate	0.772	0.444	0.099	0.225	3.668×10^{-5}
H11	glucose-6-phosphate	0.464	0.345	0.027	0.310	2.099×10^{-4}

Growth of *L. monocytogenes* on 8 carbon sources, (N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, ∝D-glucose, D-mannitol, ß-methyl D-glucoside, D-psicose, salicin, and xylitol) achieved a maximum asymptote above 2.0 at 30°C. Any carbon source that reached a maximum asymptote >1 also had a positive regression line slope for growth at 8°C. If the maximum asymptote was <1 for *L. monocytogenes* growth at 30°C, the resulting slope of the regression line at 8°C was negative. The y-intercept was ≤0.5 for the organisms growth on all carbon sources examined at 8°C.

L. monocytogenes cultured at 30°C resulted in a hyperbolic growth pattern, therefore only the initial part of the hyperbolic curve was used to calculate the slope at this temperature for comparison to the 8°C data. Growth at the two temperatures was compared for each well and the differences calculated. Growth at the two temperatures was significantly different (p=0.05) using the Sign Test. The differences between the observed data at the two temperatures were examined by calculating relative frequencies for groups of wells with similar slopes. These frequencies are given in Table IX. The wells are ranked in descending order of observed growth at the two temperatures. The carbon source wells listed in group one for growth at 30°C correspond to 78% of the wells in group one for growth at 8°C.

Table IX: Relative frequencies of the observed data condensed to 5 data classes.

Group	Wells	Slope Range	Number	Relative Frequencies
	30°C			
1	H2, B7, F6, E5, G12, D7, H6, E1, C2, H11, A4, C10, C4	0-0.0900	13	0.433
2	H1, E4, H3, H8, A12	0.0901-0.1800	5	0.167
3	D4, D1, E2, H5	0.1801-0.2700	4	0.133
4	B4, A11, D8, C5, B5	0.2701-0.3600	5	0.167
5	A10, B11, B3	0.3601-0.4500	3	0.100
	8°C	**($\times 10^{-4}$)**		
1	A12, C10, D7, H1, C2, H2, E1, A4, E5, B7, H3, H6, H8, C4, E4, C5, H11, H5	5-3.000	18	0.600
2	A11, F6, D4, A10, G12	3.001-11.000	5	0.166
3	E2, B11	11.001-19.000	2	0.067
4	B3, D1	19.001-27.000	2	0.067
5	B5, B5, D8	27.001-35.000	3	0.100

The differences between the growth of *L. monocytogenes* at the 2 temperatures was significant, which was not unexpected, as growth and respiration are chemical reactions which progress at a diminished rate at a lower temperature of incubation. This finding corresponds to the results of the effects of storage temperature on *L. monocytogenes* in coleslaw in the Phase I.

In examining the differences between the growth of *L. monocytogenes* at the 2 temperatures, *L. monocytogenes* did not show a preference for a particular group of carbon sources at 8°C versus the optimum temperature for growth, 30°C. Table IX shows the carbon sources that support the highest growth slopes at 8°C are also in the highest frequency groups at 30°C. The wells do not correspond 100% in each of the 5 slope groupings for the 2 temperatures but no particular carbon source group (for example: simple sugars, sugars that contain nitrogen, or amino acids) was preferred. All carbon source groups are utilized at both temperatures.

DISCUSSION

Modified atmosphere treatment of fruits and vegetables is a widely accepted technology for use on minimally processed fruits and vegetables (6, 15, 19, 20). In the first phase of this experiment, results indicated that modifying the atmosphere in packaged coleslaw was not effective in inhibiting the growth of *L. monocytogenes* (Table III). Berrang *et al.* (7) found that concentrations of 3-10% of CO_2 had no effect on *L. monocytogenes* growth on controlled atmosphere vegetables as compared to growth in air stored vegetables. All vegetables supported growth of *L. monocytogenes* at 15°C. *L. monocytogenes* populations ranged from 10^6-10^9 cfu/g, which occurred 6 - 10 days after the start of incubation, before being deemed unfit for human consumption due to quality deterioration. Beuchat and Brackett investigated *L. monocytogenes* growth on iceberg lettuce stored at 5 and 10°C in a modified atmosphere of 3% O_2 and 97% N_2 versus ambient air packaging (9). Again, *L. monocytogenes* grew on the lettuce reaching populations of 10^8 cfu/g after 10 days of storage. Phase I results support these findings as both mean *L. monocytogenes* and total bacteria counts increased approximately 2 log cycles by day 6 of storage from the start of incubation and then remained relatively constant (Table VI). Populations of 10^6 cfu/g of *L. monocytogenes* and approximately 10^8 cfu/g of total bacteria were obtained before any obvious spoilage indicators were noted in the sensory observations (TableV).

Sensory attributes of the coleslaw deteriorated at 8°C after 6 d storage in gas

flush packages (Table V). Gas flush packages had a slightly higher mean CO_2 level (however, not significantly) but this may have led to the earlier deterioration of the cabbage tissue. In comparison, coleslaw in ambient packages did not deteriorate until 9 d of storage even though the packages received the same inoculum levels. This could possibly be due to mechanical damage from drawing a vacuum on the packaging during the gas flush procedure, which could enhance deterioration of the cabbage and carrot tissue. A microbial study on the shelf life of cabbage and coleslaw revealed that coleslaw deteriorated over similar storage regimes as used in phase I of this study, leaving the authors to conclude that the deterioration was primarily caused by physiological breakdown of plant tissue rather than by microorganisms (73).

The optimal O_2 levels for vegetables, in general, are between 2% and 5% and they can tolerate up to 20% CO_2 before deterioration of sensory characteristics appear (9). The mean package atmospheres in this study for O_2 ranged from 1.4% - 4.3% and the range for CO_2 was 17-23% as reported in Table VI. Since the package material used for the coleslaw was permeable to gas transfer, the modified atmosphere packages did equilibrate to ambient air treatments. *Ballantyne et al.*(3) also found in two different studies that shredded lettuce and broccoli florets will modify the atmosphere naturally by respiratory processes (3, 4). In phase I, the coleslaw packages remained aerobic for the duration of the study. However, low oxygen concentrations or high carbon dioxide levels, above the tolerance of the coleslaw, can induce anaerobic respiration, which can cause the production of off-odours or flavours (3, 20).

Another benefit of using modified atmosphere is supposed to be the extension of produce shelf-life. In phase I, packages were gas flushed with 5% O_2, 5% CO_2 and 90% N_2, which has been reported as a successful combination to double shelf-life of shredded lettuce and fresh bell peppers (4, 14). No such benefits were obtained by using this preservation method in coleslaw (Table V).

Even though vegetables can only tolerate up to 20% CO_2 (9), extremes in CO_2 levels were examined for their ability to prevent growth of *L. monocytogenes*. Studies on shredded cabbage stored at 5°C under a modified atmosphere of 70% CO_2 / 30% N_2 versus normal atmosphere indicated *L. monocytogenes* counts increased gradually over storage time by 1 log in both atmospheres and then dropped sharply after 13 days (70). This coincided with sharp decreases in cabbage pH and the development of spoilage characteristics. The extreme CO_2 atmosphere also was not effective against the growth of *L. monocytogenes*, and in addition caused deterioration in the quality of the cabbage at a faster rate than phase I even though the coleslaw was intentionally inoculated with a high inoculum level.

In this study, coleslaw was inoculated with two levels of *L. monocytogenes*, 10^4 and 10^6 cfu/g, yet inoculum level had no significant effect on the average increase in number of *L. monocytogenes* recovered from the coleslaw (Table II). The higher inoculum gave *L. monocytogenes* no competitive advantage against the inherent flora. The mean total bacteria counts were also not significant since the higher level of *L. monocytogenes* inoculum (10^6/g) did not result in higher mean total bacterial counts in

the coleslaw.

Low temperature storage is another method used to prevent spoilage by bacterial organisms. A significant effect on the mean counts of both *L. monocytogenes* and total bacterial counts were evident with the slight increase in temperature (Table IV). A similar study of *L. monocytogenes* on ready-to-serve lettuce (110) found that the *L. monocytogenes* counts increased by several log cycles during 14 d of storage at 5°C and 12°C. Previous authors reported that storage temperatures below 7°C effectively suppressed bacterial growth (3, 73). The ability of *L. monocytogenes* to grow at low storage temperatures make it particularly important to chilled foods. Seeliger and Jones (104) and Junttila *et al.* (69) reported that the low temperature limit for growth of *Listeria* species is 1°C. As well, Beuchat *et al.* indicated that *L. monocytogenes* can proliferate on refrigerated (5°C) raw cabbage (10). Temperature abuse during the distribution chain of coleslaw, like that reported by Parry (73), where the product left for prolonged periods at ambient temperatures of +15°C (after unloading and prior to transfer to refrigerated storage) could result in an increased public health hazard. Coleslaw has a retail shelf-life greater than 6 days. Again, phase I showed no obvious deterioration of package quality until after 6 days of storage (TableV), even though *L. monocytogenes* had reached the level of 10^6 cfu/g. Therefore refrigeration by itself is not sufficient to guarantee microbiological safety of refrigerated foods, since it is accepted that at some point during distribution or consumer handling temperature abuse can occur(59, 93). To eliminate the potential risk to the consumer of listeriosis a shorter

shelf-life may be necessary, even though the coleslaw may appear to be acceptable.

The second phase of this study examined the growth of a pure strain of *L. monocytogenes* on various carbon sources by utilizing a Biolog® MicroPlate. Studies have been done on carbohydrate fermentation by *L. monocytogenes* (85). Under anaerobic conditions only hexoses and pentoses supported growth whereas in aerobic conditions, glucose, rhamnose and lactose were preferred (85). Growth on the sugars in Biolog® was positive for glucose at both 8°C and 30°C, but was negative for both L-rhamnose and α-D-lactose, which is in agreement with the Biolog® expected growth reported in the bacterial identification library program.

The differences between the observed growth on the various carbon source wells and the expected response according to the Biolog® software library program could possibly be due to the actual strain of *L. monocytogenes* cultured. The software library for the identification of *L. monocytogenes* is for an environmental strain cultured most effectively at 30°C for optimum growth results. The Biolog® manual recommends an incubation temperature of 35°C for human, veterinary and food isolates, such as the *L. monocytogenes* Scott A strain used in this study. The temperature of 30°C was selected for comparison to 8°C as this temperature was more comparable to hot summer vegetable growing days in July and August in Nova Scotia than the higher temperature of 35°C.

As well, growth factor requirements can change with temperature (123). For example, *Escherichia coli* requires glutamic acid and nicotinamide for growth at

44°C(97). *L. monocytogenes* utilized twice as many carbon sources when the incubation temperature was elevated to 30°C from 8°C. Growth for N-acetyl-D-glucosamine and N-acetyl-D-glucose was positive for both 8°C and 30°C which is not surprising as this is one of the components of *L. monocytogenes* that is isolated from cell walls (85).

Other possible causes for variation between expected growth and the observed growth could be due to the initial broth (tryptic phosphate broth) used to culture *L. monocytogenes*. This media gave a very low raw optical density reading (0.002) in the A1 well, which the colour density in every other well is referenced against to designate the growth pattern. This could explain why 8% of the carbon sources were designated negative versus positive, as expected by the library program. The Biolog® program has a 40% change threshold for determination of whether a well is designated positive. For the purposes of this study, only the raw optical densities were used and the mean of the growth in 6 replicate plates at 24 hours was used to determine whether a well was positive or negative.

The difference between temperatures for growth on the carbon sources that were positive was highly significant (p=0.05). The pattern of growth at 30°C was hyperbolic whereas at 8°C was linear. When the initial part of the growth curve at 30°C was analysed with the same equation as 8°C growth, the frequency distributions were very similar. Therefore carbon sources that cultured *L. monocytogenes* effectively at 30°C also cultured *L. monocytogenes* at 8°C but at a much lower rate of growth.

Information on the preferred carbon sources for the growth of *L. monocytogenes*

at chilled temperatures can be applied to the formulation of foods susceptible to growth of this organism. For example, an alternate sugar could be used for sweetening a food product such as L-fructose instead of sucrose. Duffy *et al.* study showed no difference between the growth curves obtained for *L. monocytogenes* in pure or food culture systems (36). Therefore the Biolog® system has the potential to model microbial growth of *L. monocytogenes* as well as other microorganisms to assess how organisms are affected by factors such as preservatives or acidulants in food. Relative frequencies of the slopes, shown in Table IX, can determine the optimum carbon source for growth at a particular temperature. Biolog® could be used to evaluate the effectiveness of antagonistic microorganisms. The use of food-grade microorganisms and/or their by-products as food additives is being investigated for their ability to reduce or eliminate undesirable microorganisms in a food such as coleslaw. Microorganisms with potential antagonistic activity include lactic-acid bacteria, propionic-acid bacteria and possibly yeasts and acetic-acid bacteria (21).

A study of the growth of *L. monocytogenes* in experiments with meat products revealed that the growth rates at 7°C versus 30°C were similar and that the growth temperature only affected the lag phase (12,16). This also agrees with statistical analysis of the results obtained in the coleslaw in Figure 6, with no significant difference in growth rate at different storage temperatures.

In other studies, *L. monocytogenes* was either incapable of multiplying, or showed a very slow increase in levels and this was attributed to differences in the

composition of the food products(12,42,112). Biolog® has shown that the carbon source greatly affects the growth of *L. monocytogenes.* In coleslaw not only the presence of other organisms, but also the composition of the food affects the growth of *L. monocytogenes*. The growth of enterococci and Gram negative organisms has been recognized for their inhibition of the growth of *Listeria spp.*, therefore, selective media for *Listeria* contain antibiotics to suppress these species. It has also been reported that at low temperatures *Pseudomonas* spp. stimulate the growth of *Listeria*(36). *Pseudomonas* and Gram negative bacteria were the predominant isolates from the identification tests on coleslaw reported in Table VII. The Biolog® MicroPlate could be utilized to model the effect of *Pseudomonas* on the growth of *L. monocytogenes* in a mixed inoculum culture, in future research.

CONCLUSIONS

The growth of *L. monocytogenes* in coleslaw when subjected to modified atmosphere was not significantly affected, regardless of inoculum level. Consequently the higher inoculum gave *L. monocytogenes* no competitive advantage against the inherent flora.

The higher initial inoculum level of *L. monocytogenes* does not result in higher overall levels of *Listeria*, but incubation at higher temperature does lead to higher *Listeria* levels. This increase in population was obtained before any sensory deterioration occurred in the package that would warn the consumer of spoilage. Based on the above findings, further studies are needed to examine the competitiveness of *L. monocytogenes* at a much lower level of inoculum more representative of possible contamination levels from the environment. Application of this data to the storage of perishable foods may be important. If the storage temperature is raised only slightly conditions may become more favourable for extensive growth of this pathogen.

Consumers should be alerted to the consequences of temperature abuse on the growth of food pathogens especially when consumption of commercially prepared, ready-to-eat fresh salads and vegetables is increasing. *L. monocytogenes'* psychrotrophic nature makes it a pathogen of great concern with respect to its potential for foodborne disease. As most healthy people are not susceptible to listeriosis, outbreaks have not been more widespread.

It is apparent from the results of the Phase II section of this study that temperature has a great influence on the growth of *L. monocytogenes* on various carbon sources. The fact that *L. monocytogenes* showed positive growth at 8°C on certain carbon sources common in many foods reinforces the need for concern about this pathogen in minimally processed refrigerated foods. Refrigeration storage alone cannot assure that the growth of *L. monocytogenes* will not occur.

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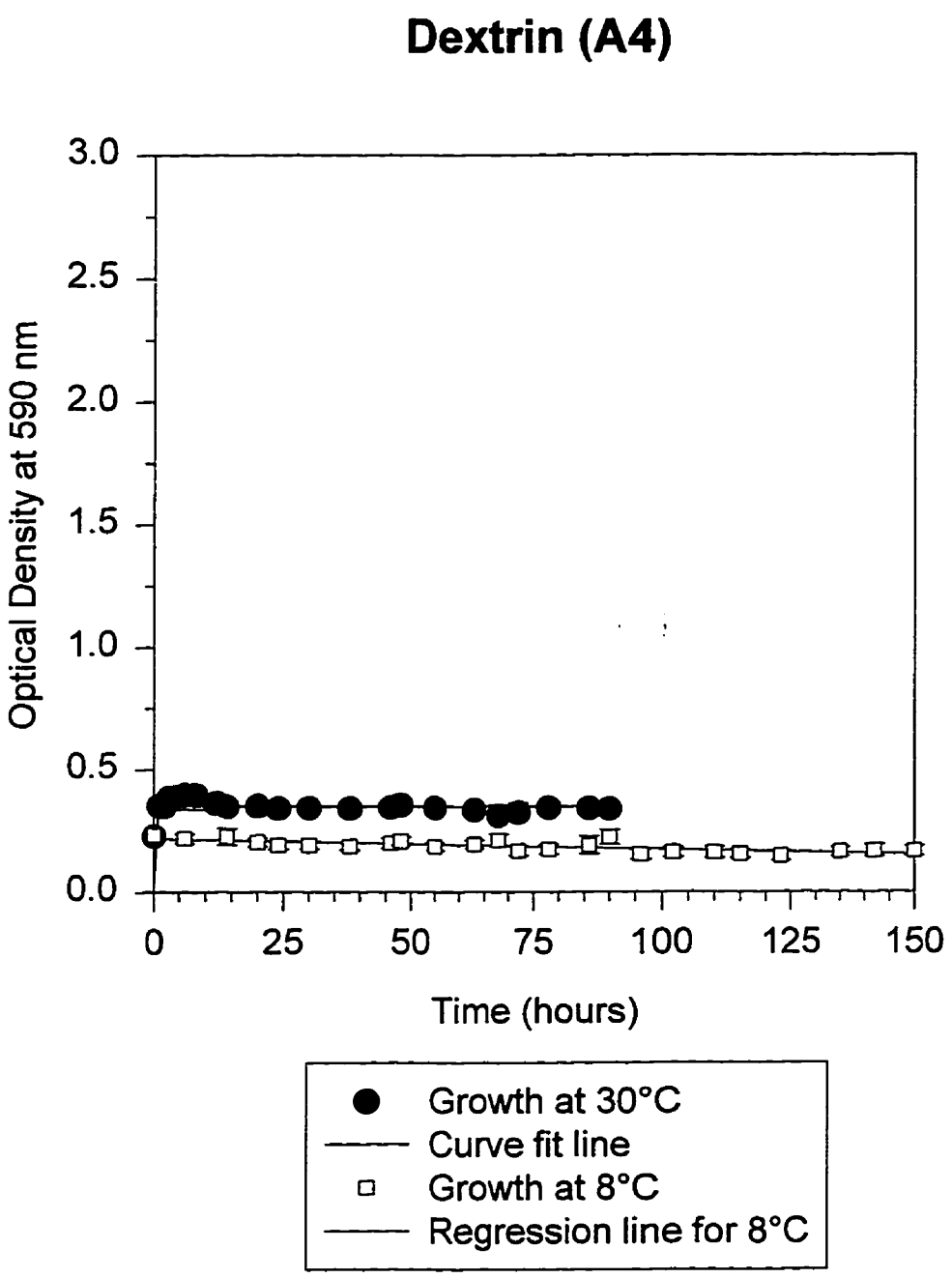
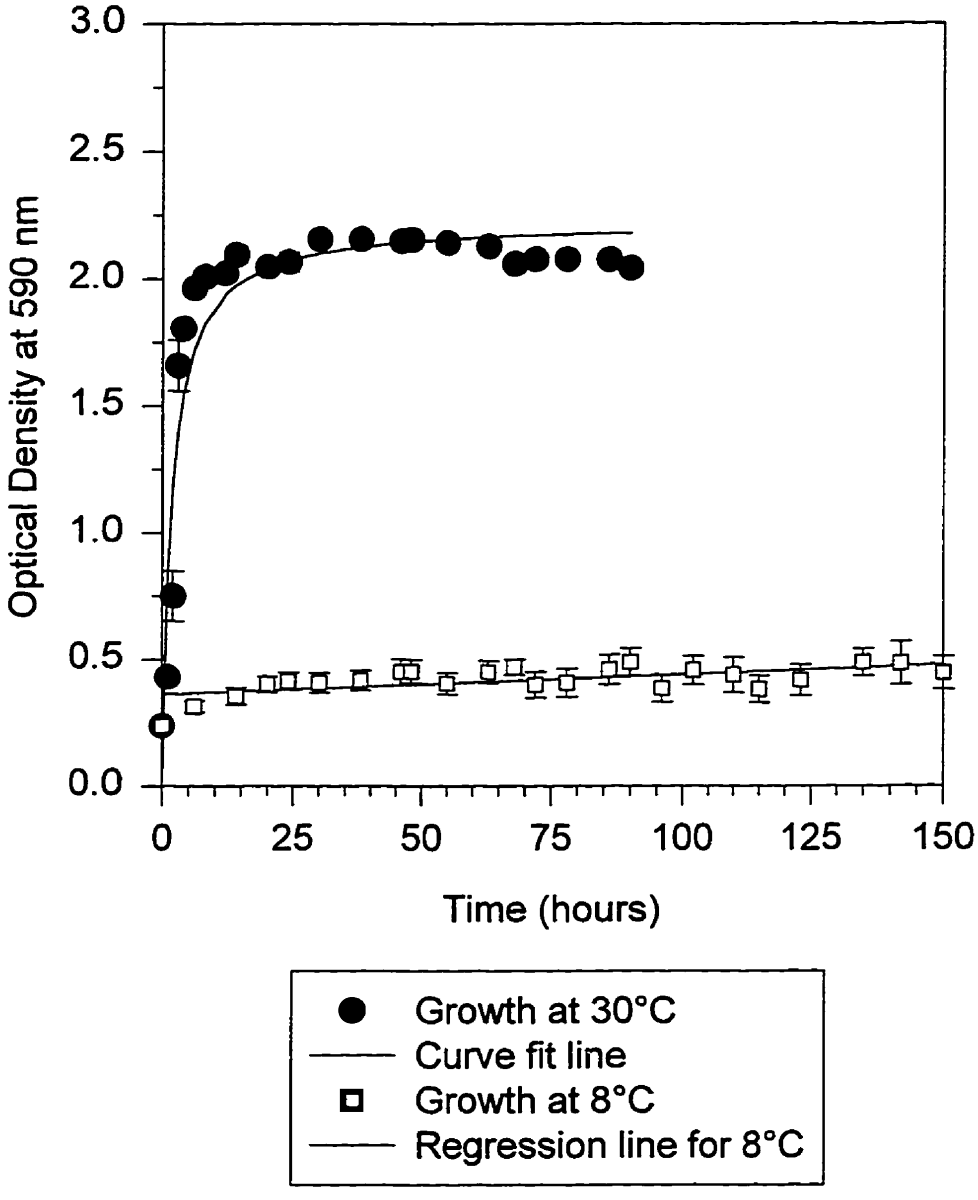
Appendix I: GP Biolog Plate

A1 water	A2 α- cyclodextrin	A3 ß- cyclodextrin	A4 dextrin	A5 glycogen	A6 inulin	A7 mannan	A8 tween 40	A9 tween 80	A10 N-acetyl-D- glucosamine	A11 N-acetyl-D- mannosamine	A12 amygdalin
B1 L- arabinose	B2 D- arabitol	B3 arbutin	B4 cellobiose	B5 D-fructose	B6 L-fucose	B7 D- galactose	B8 D-galacturonic acid	B9 gentiobiose	B10 D- gluconic acid	B11 α-D- glucose	B12 m- inositol
C1 α-D- lactose	C2 lactulose	C3 maltose	C4 maltotriose	C5 D- mannitol	C6 D- mannose	C7 D- melezitose	C8 D- mellibiose	C9 α-methyl D-galactoside	C10 ß-methyl D-galactoside	C11 3-methyl glucose	C12 α-methyl D-glucoside
D1 ß-methyl D-glucoside	D2 α-methyl D- mannoside	D3 palatinose	D4 D- psicose	D5 D- raffinose	D6 L- rhamnose	D7 D- ribose	D8 salicin	D9 sedoheptulosan	D10 D-sorbitol	D11 stachyose	D12 sucrose
E1 D- tagatose	E2 D- trehalose	E3 turanose	E4 xylitol	E5 D- xylose	E6 acetic acid	E7 α- hydroxybutyric acid	E8 ß- hydroxybutyric acid	E9 γ- hydroxybutyric acid	E10 p- hydroxyphenyl acetic acid	E11 α- keto glutaric acid	E12 α- keto valeric acid
F1 lactamide	F2 D- lactic acid methyl ester	F3 L- lactic acid	F4 D- malic acid	F5 L- malic acid	F6 methyl pyruvate	F7 mono-methyl succinate	F8 propionic acid	F9 pyruvic acid	F10 succinamic acid	F11 succinic acid	F12 N-acetyl L- glutamic acid
G1 alaninamide	G2 D-alanine	G3 L-alanine	G4 L-alanyl- glycine	G5 L- asparagine	G6 L-glutamic acid	G7 glycyl-L- glutamic acid	G8 L-pyroglutamic acid	G9 L-serine	G10 putrescine	G11 2,3-butanediol	G12 glycerol
H1 adenosine	H2 2'deoxy adenosine	H3 inosine	H4 thymidine	H5 uridine	H6 adenosine-5'- monophosphate	H7 thymidine-5'- monophosphate	H8 uridine-5'- monophosphate	H9 fructose-6- phosphate	H10 glucose-1- phosphate	H11 glucose-6- phosphate	H12 D-L-α-glycerol phosphate

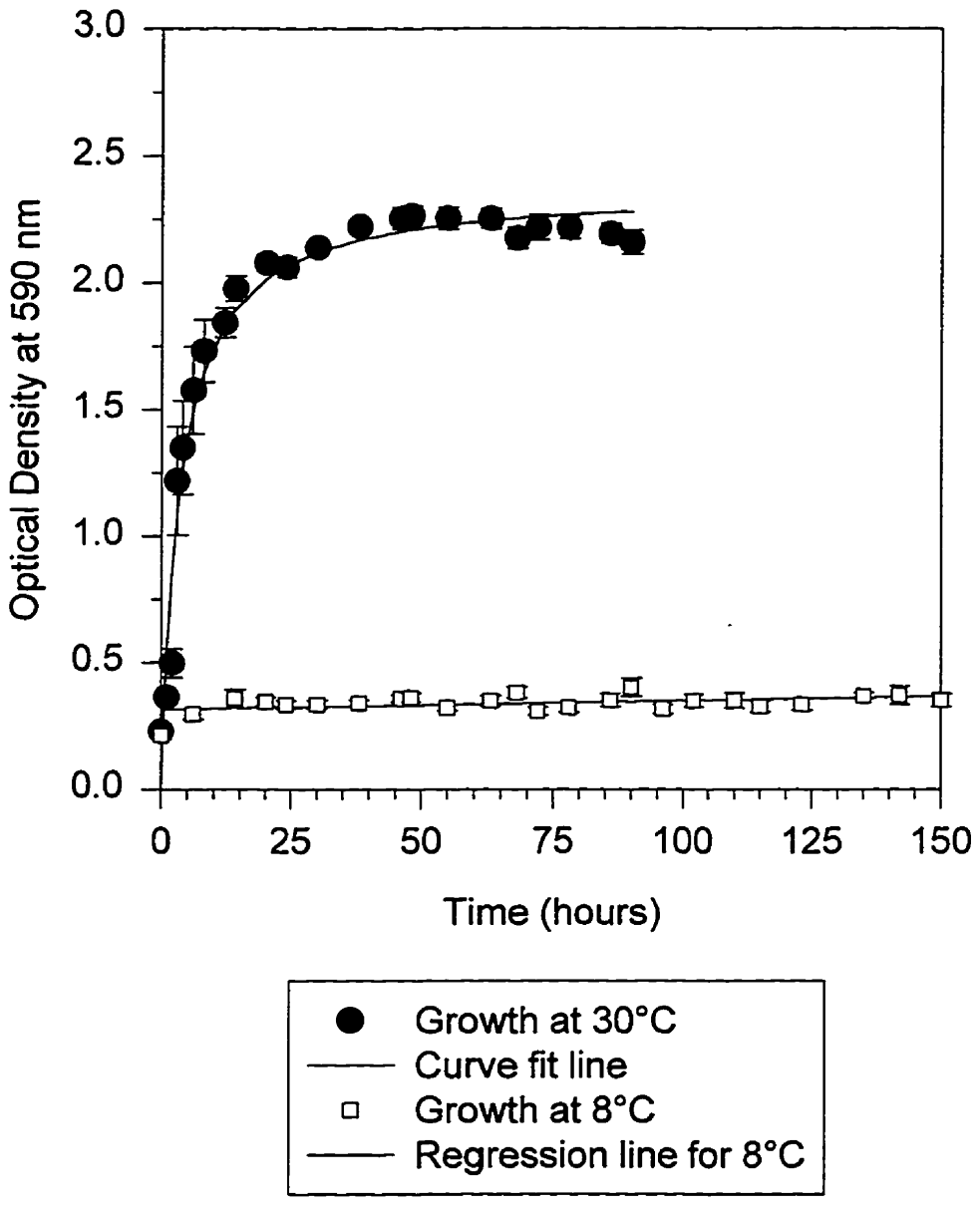
APPENDIX II. Growth of *L. monocytogenes* on various carbon sources in a Biolog® MicroPlate.

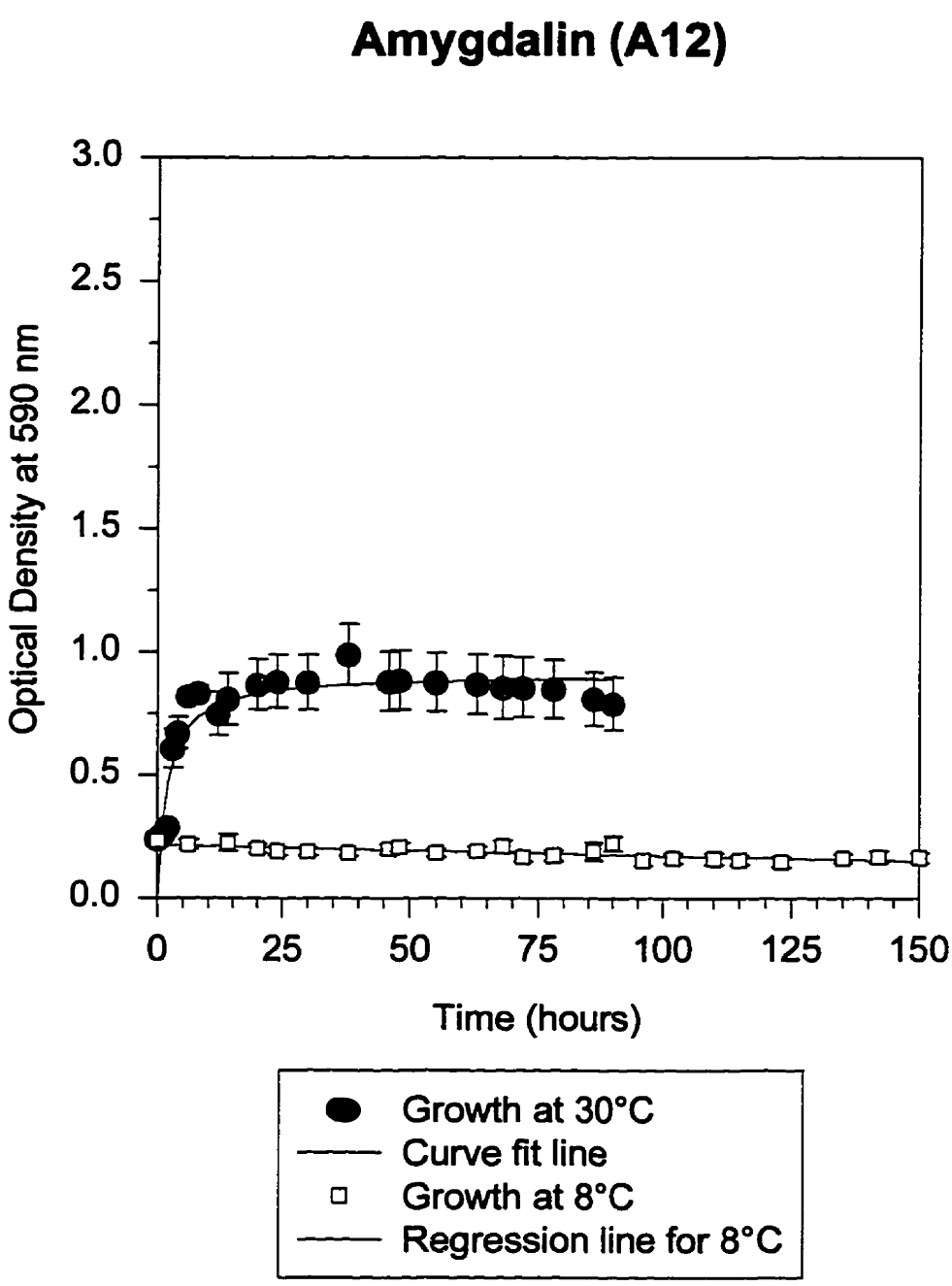

Dextrin (A4)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

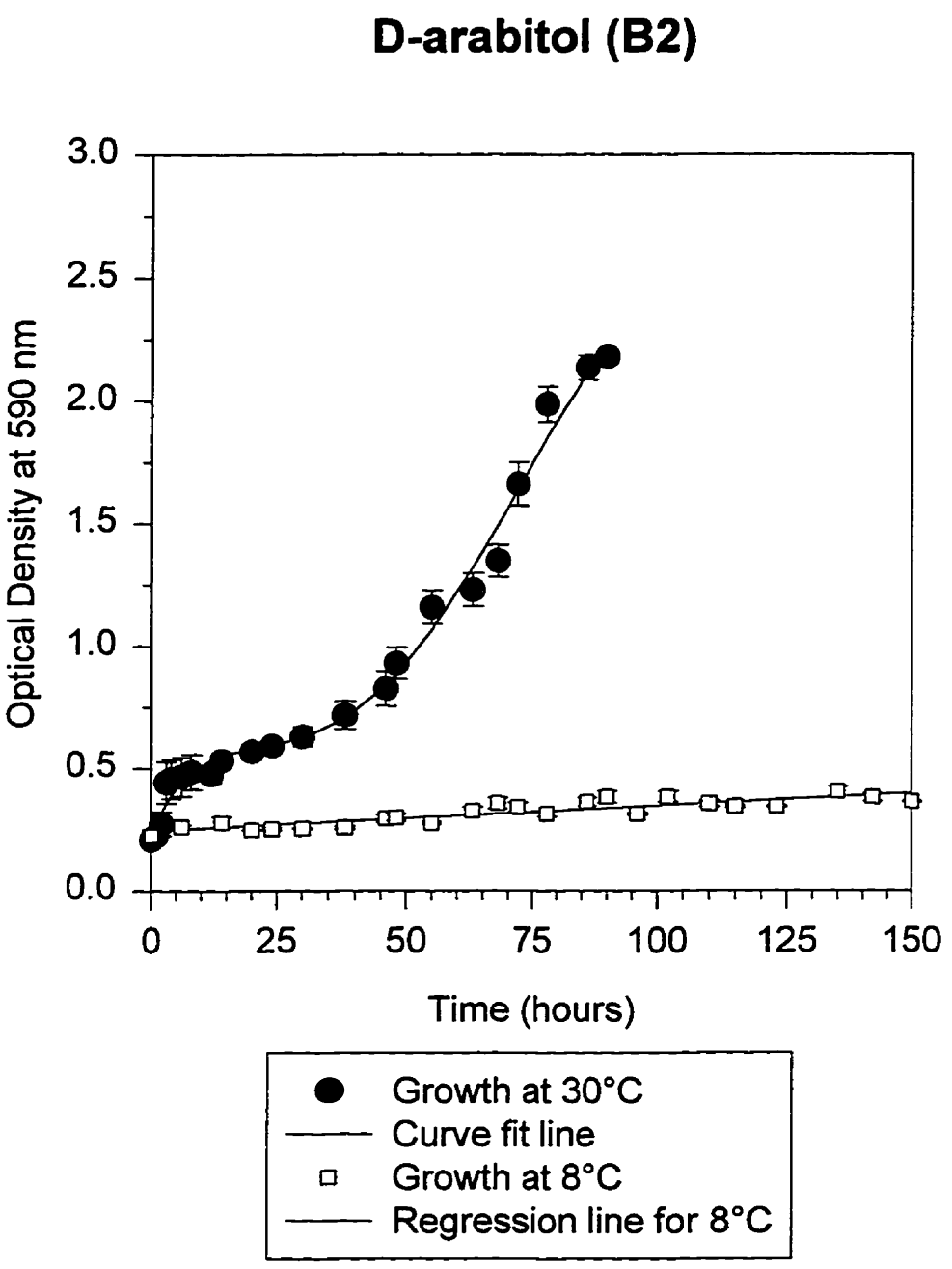
N-acetyl-D-glucosamine (A10)

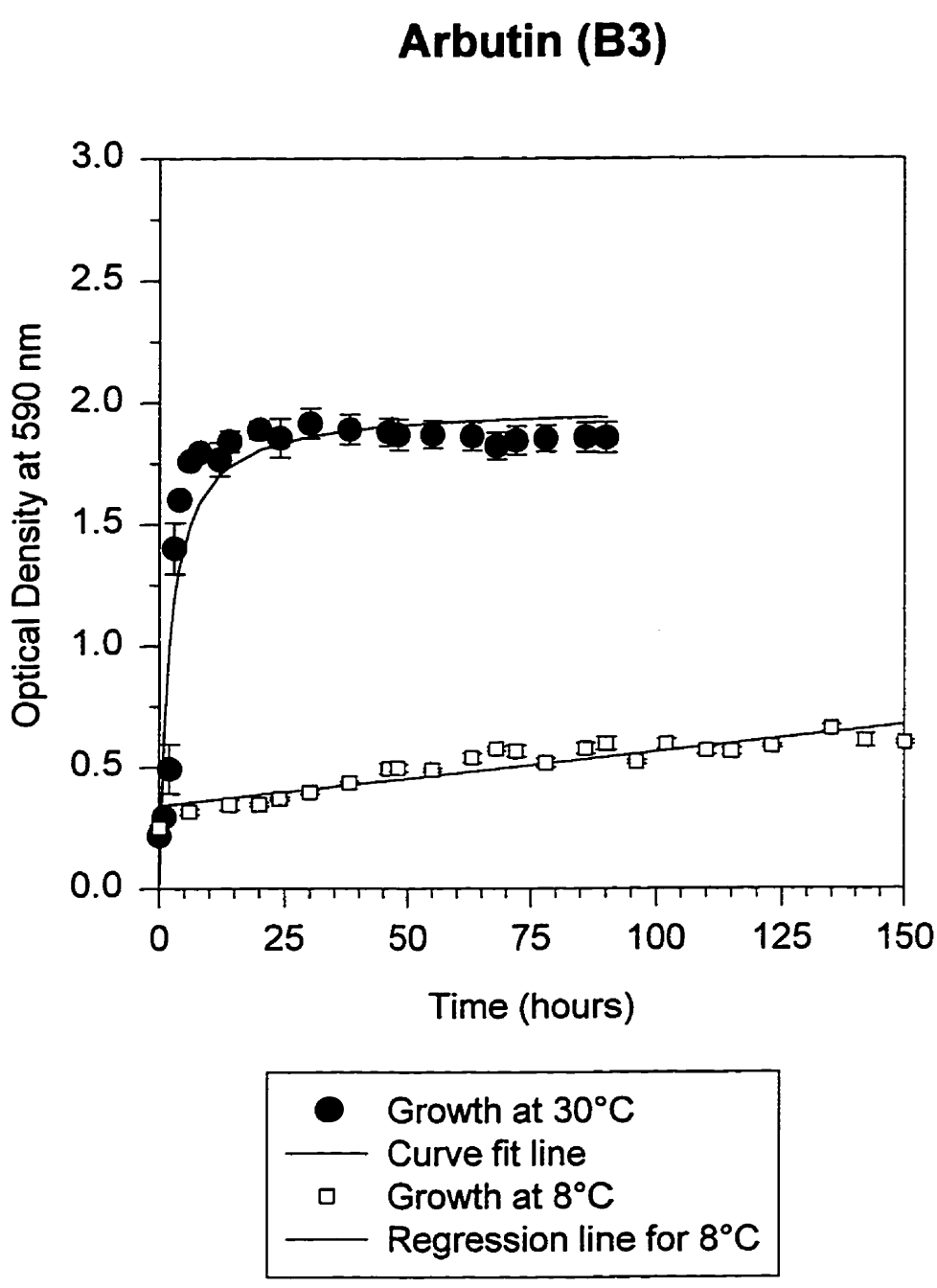


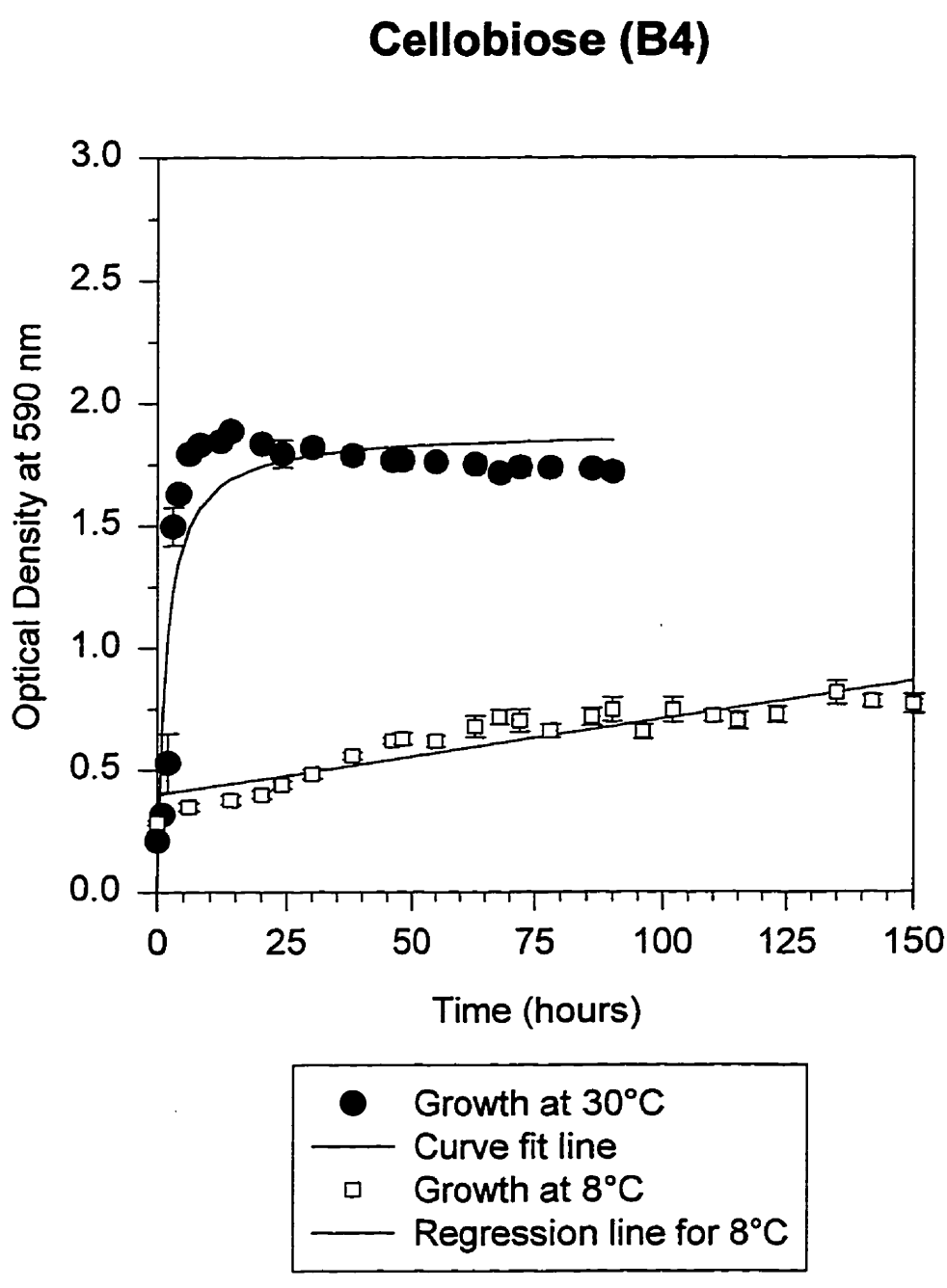
N-acetyl-D-mannosamine (A11)

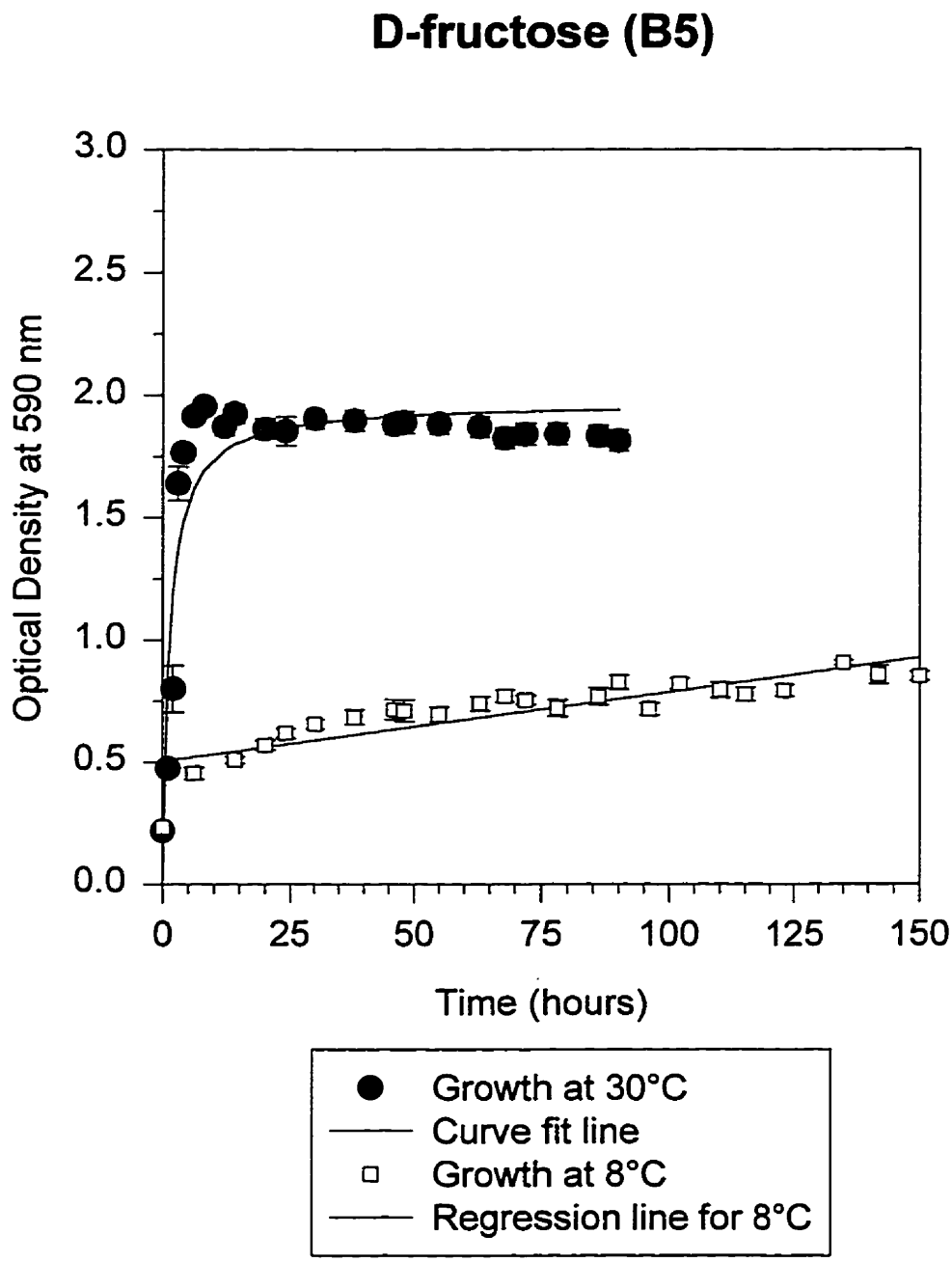


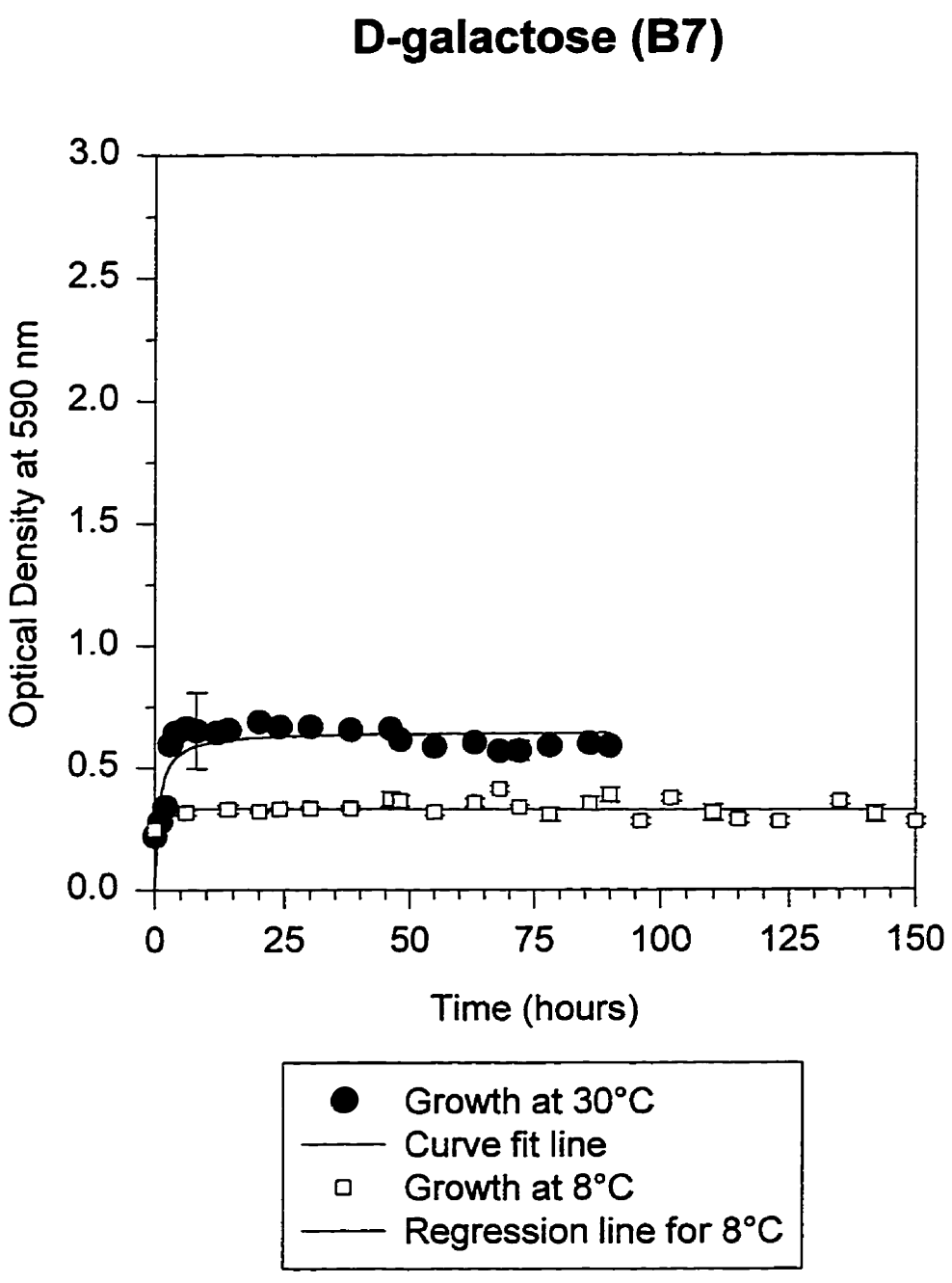

Amygdalin (A12)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

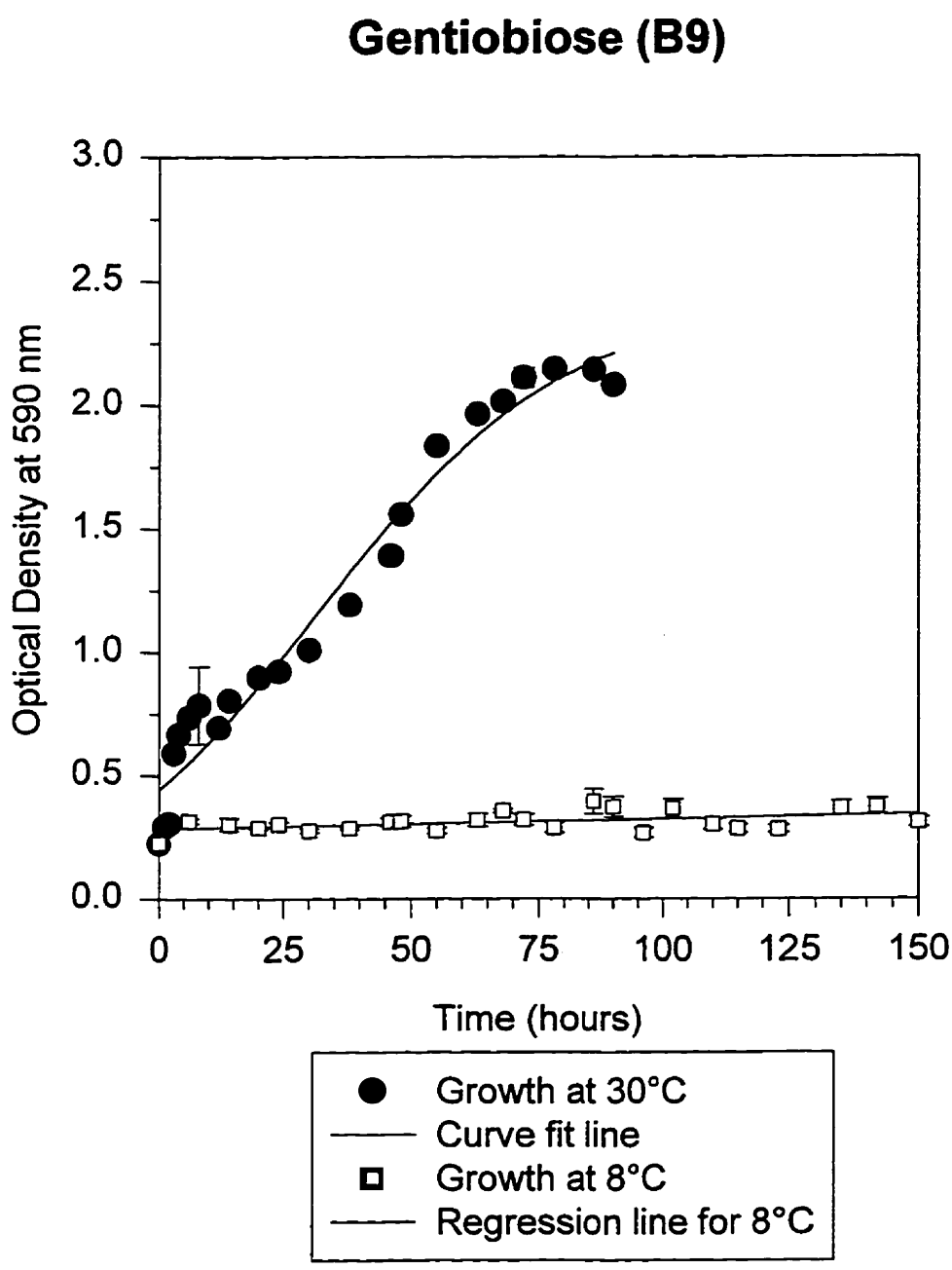

D-arabitol (B2)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

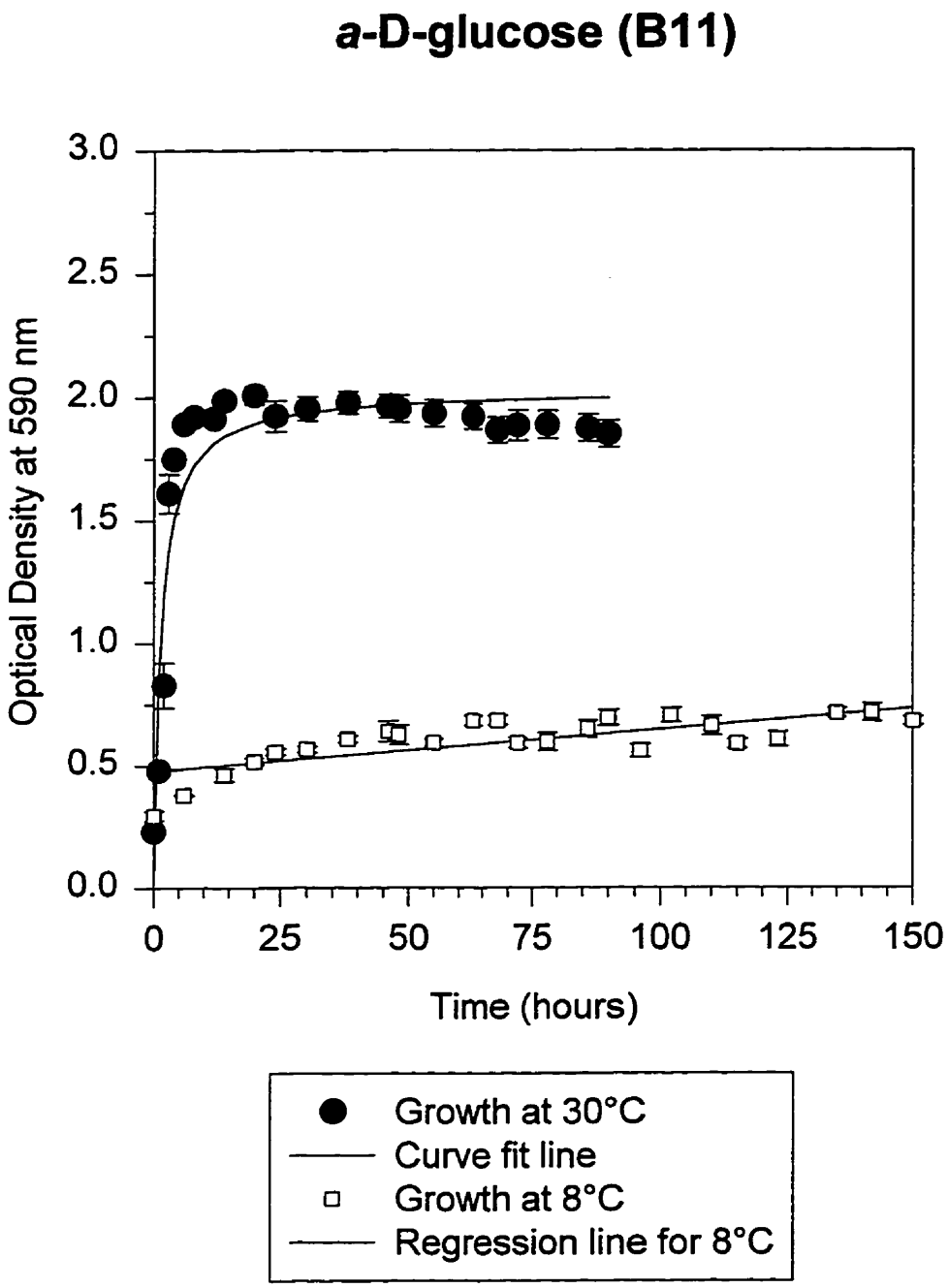

Arbutin (B3)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

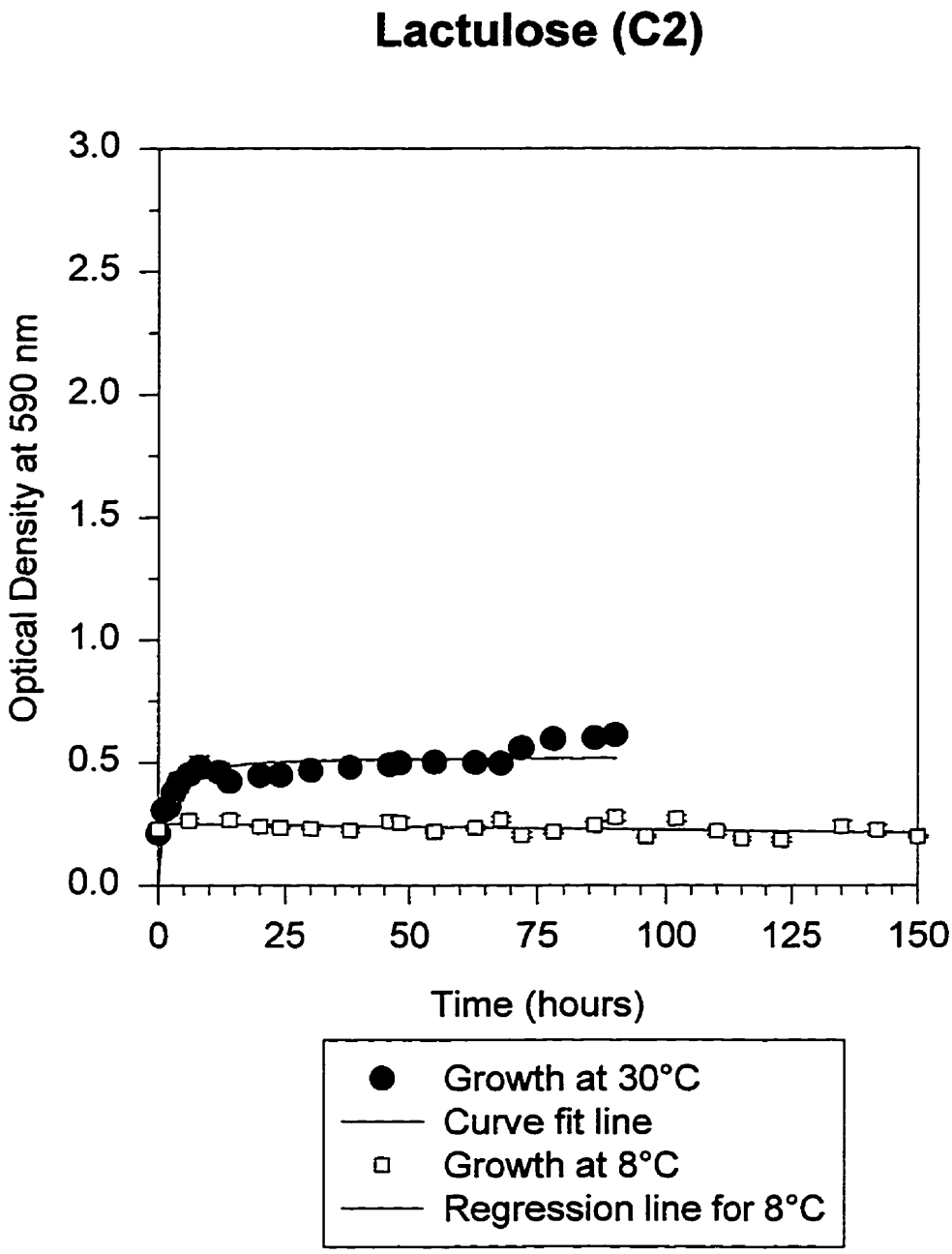

Cellobiose (B4)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density at 590 nm
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

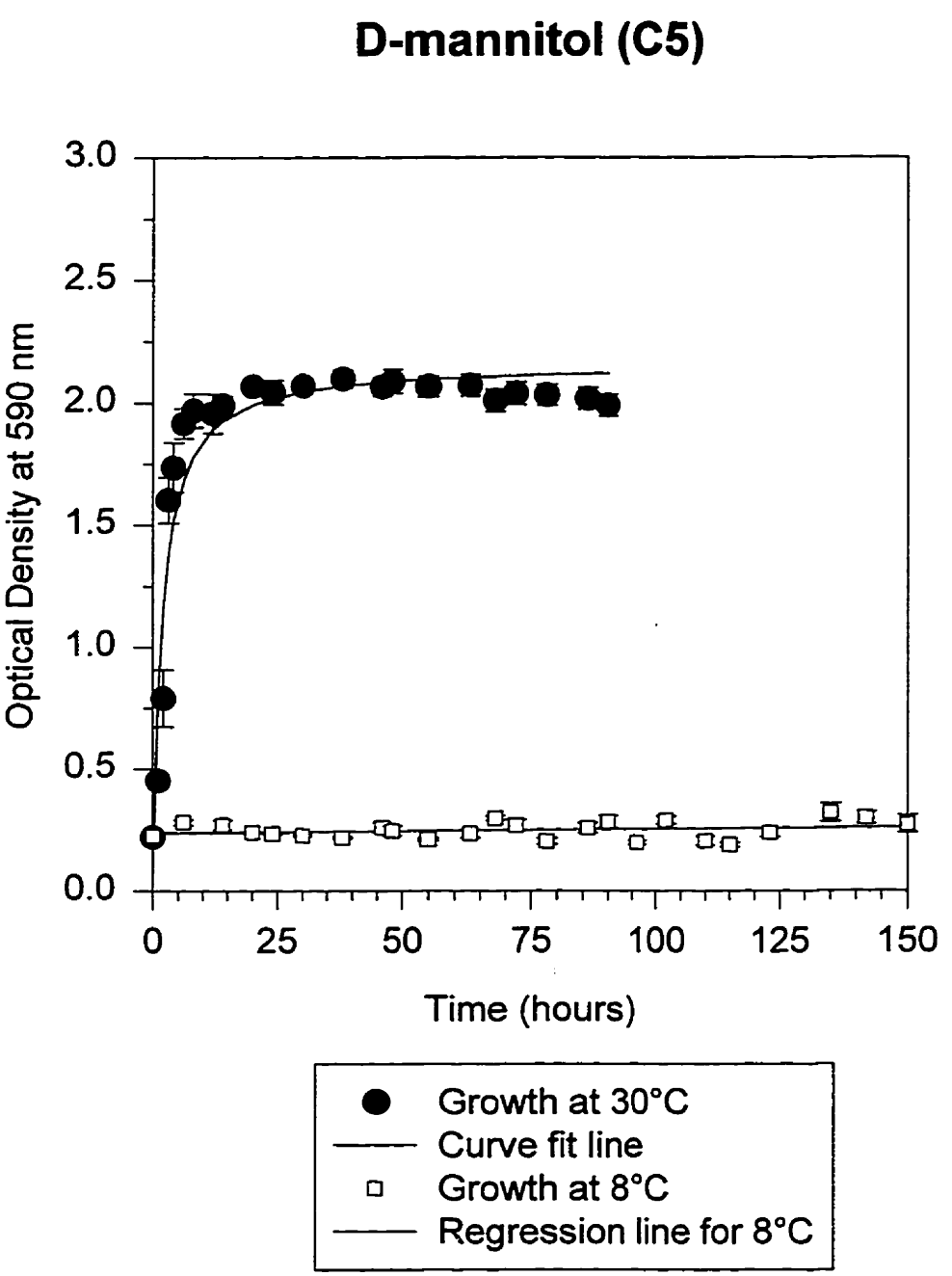

D-fructose (B5)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C


D-galactose (B7)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

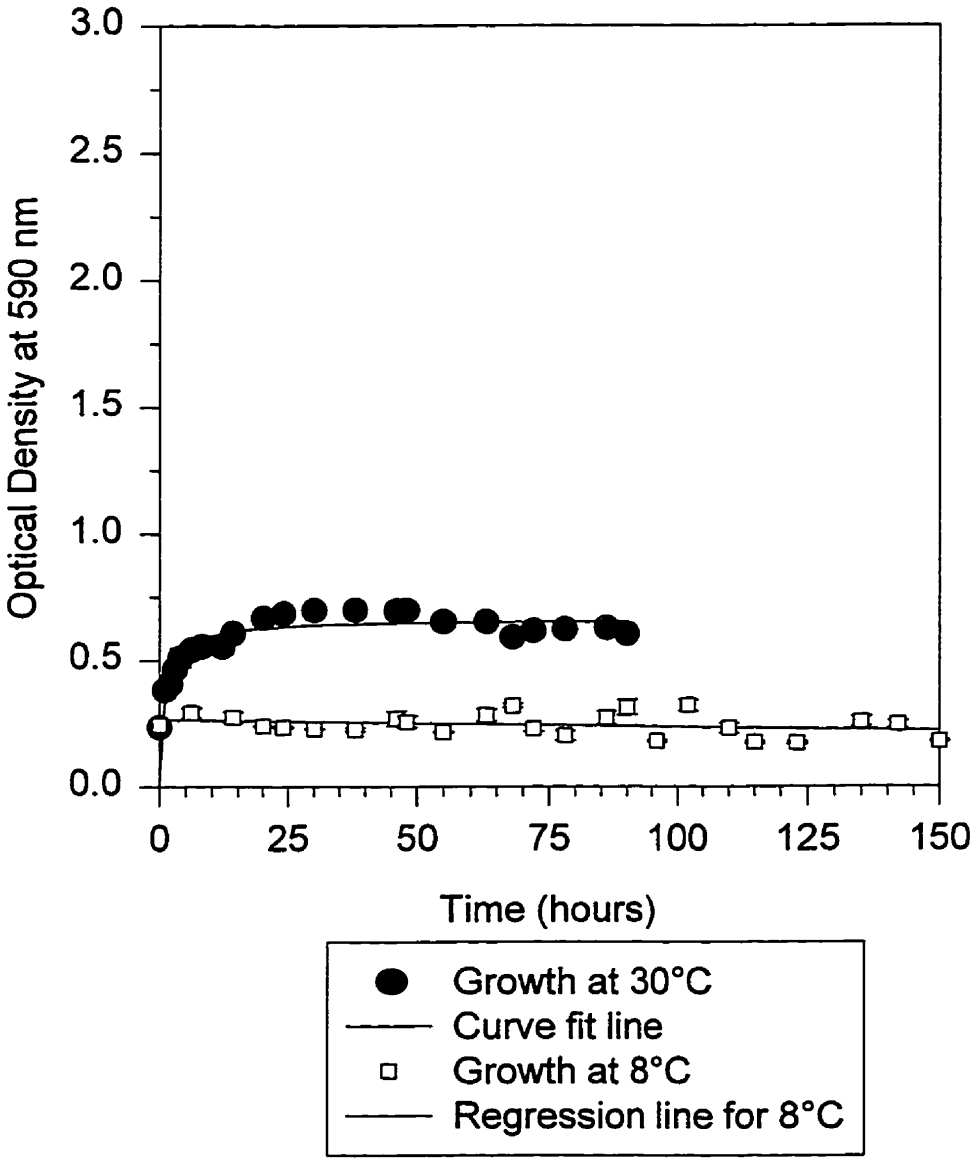

Gentiobiose (B9)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

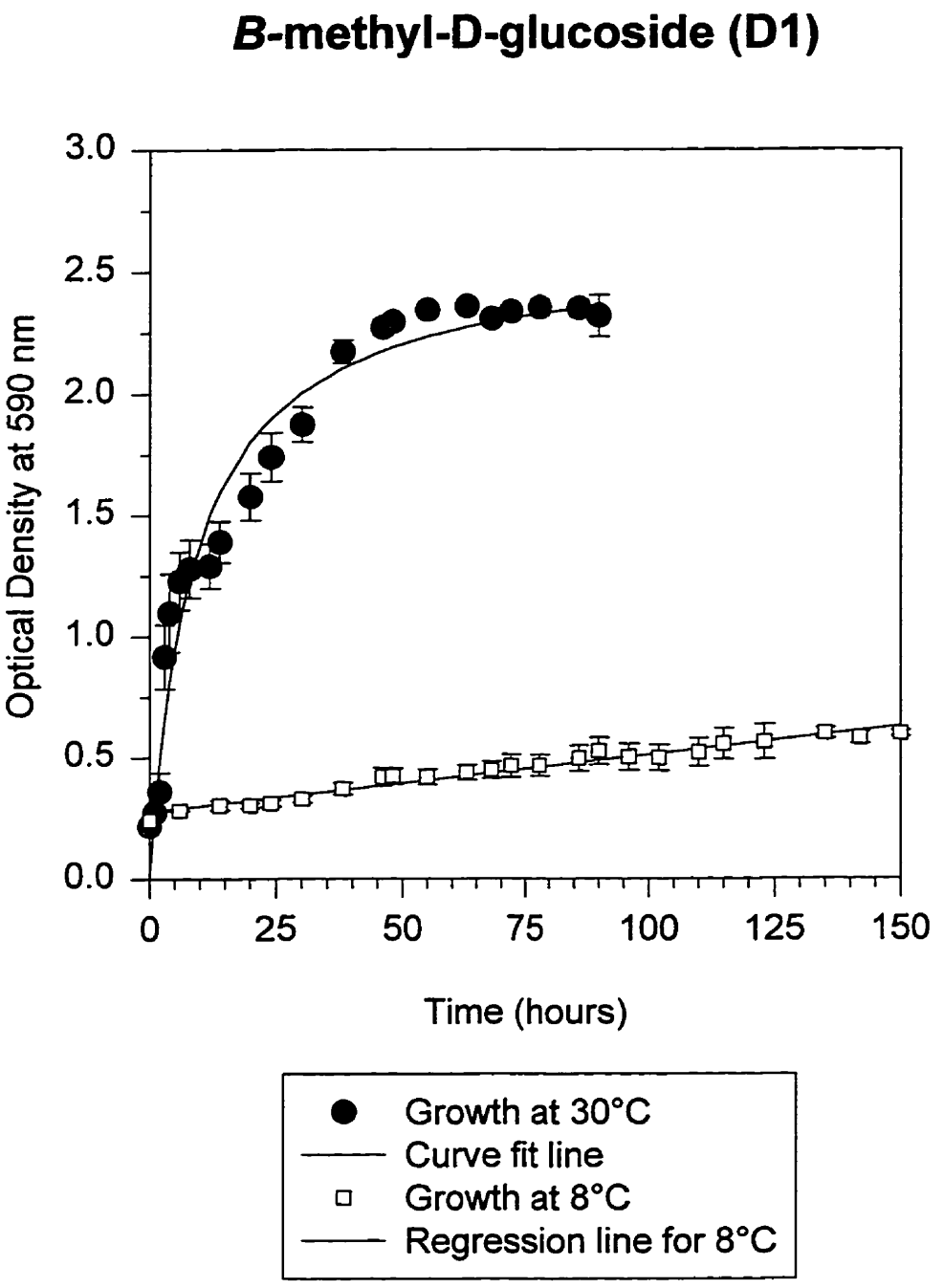

a-D-glucose (B11)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

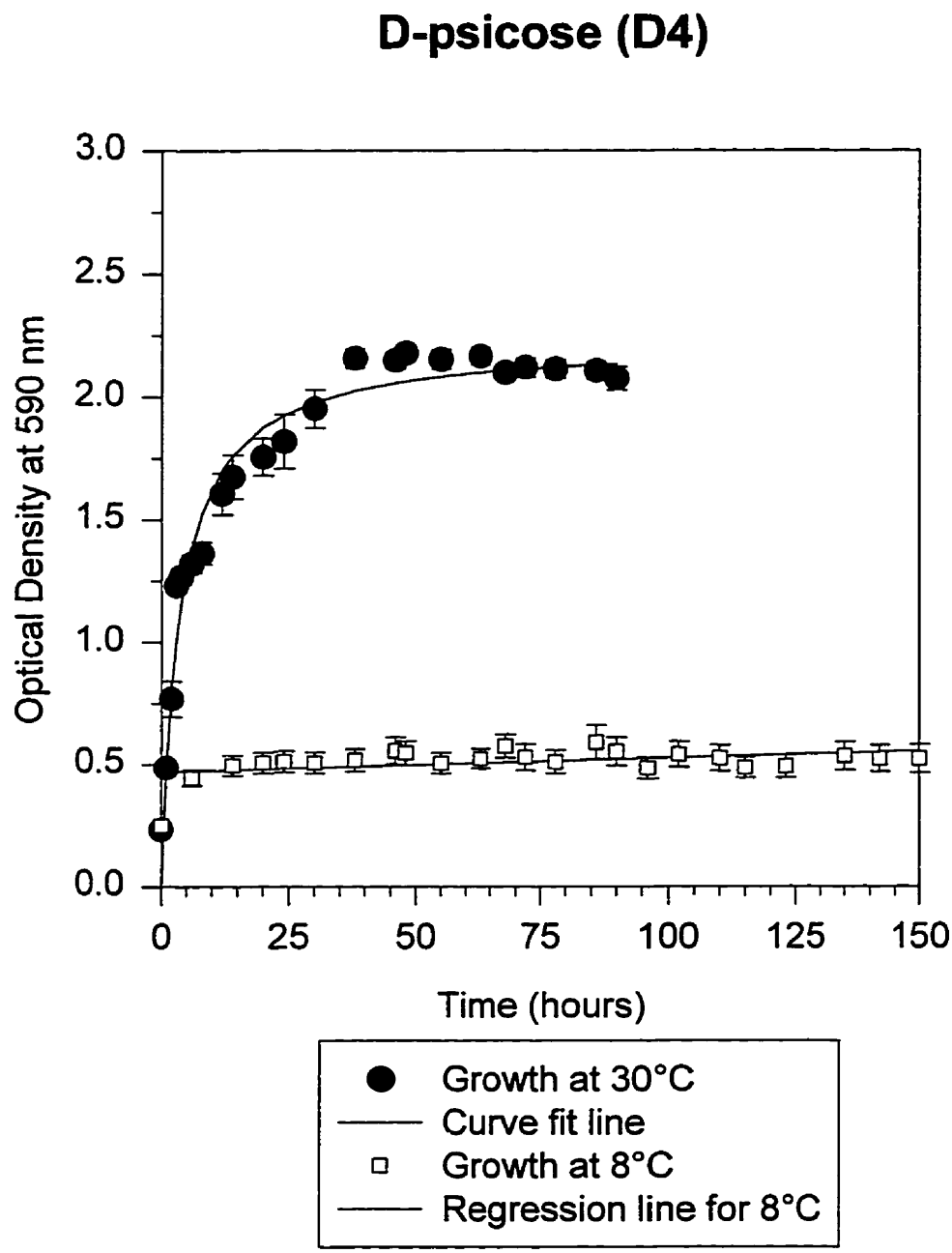

Lactulose (C2)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

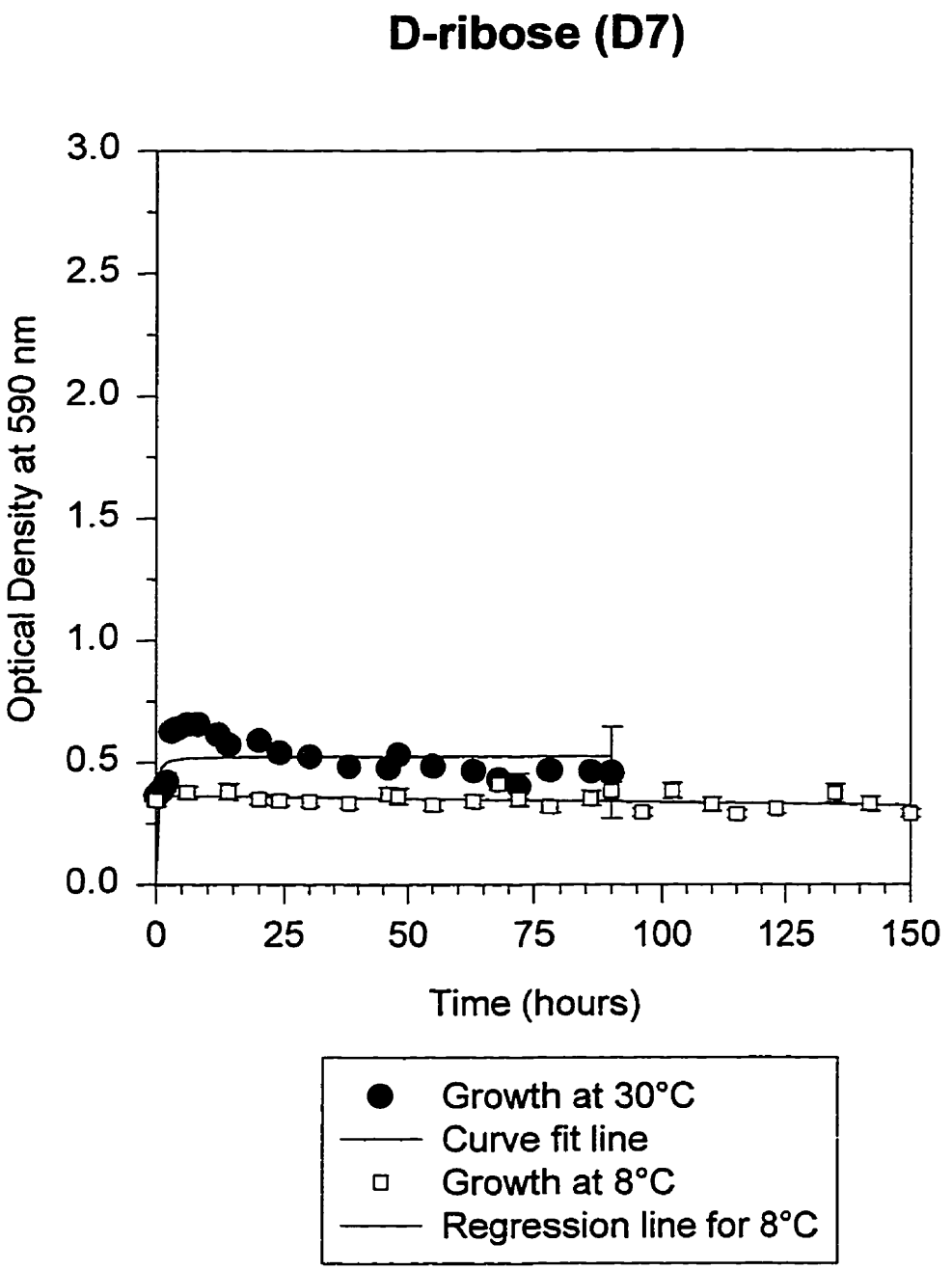

D-mannitol (C5)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

b-methyl-D-galactoside (C10)

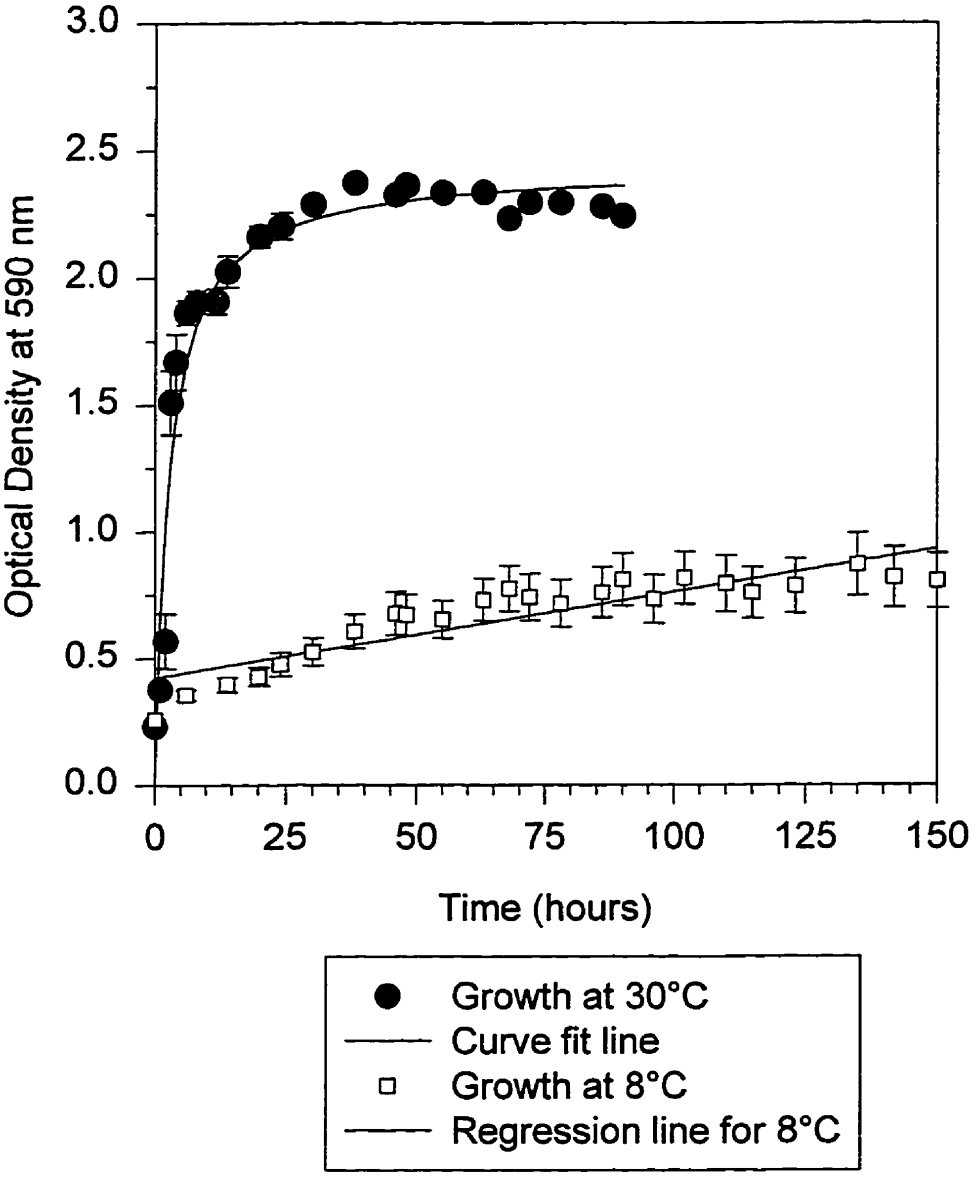


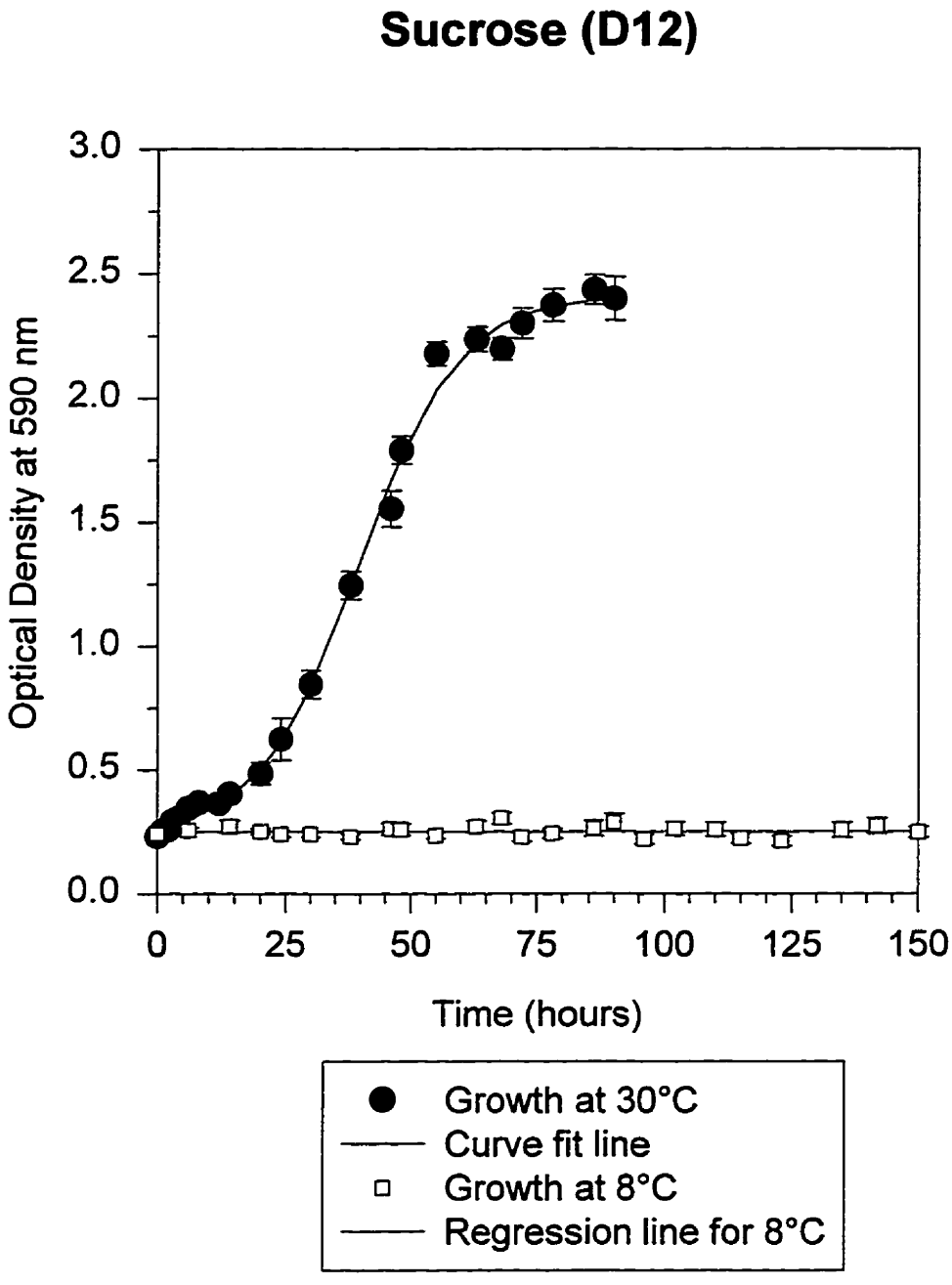

B-methyl-D-glucoside (D1)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

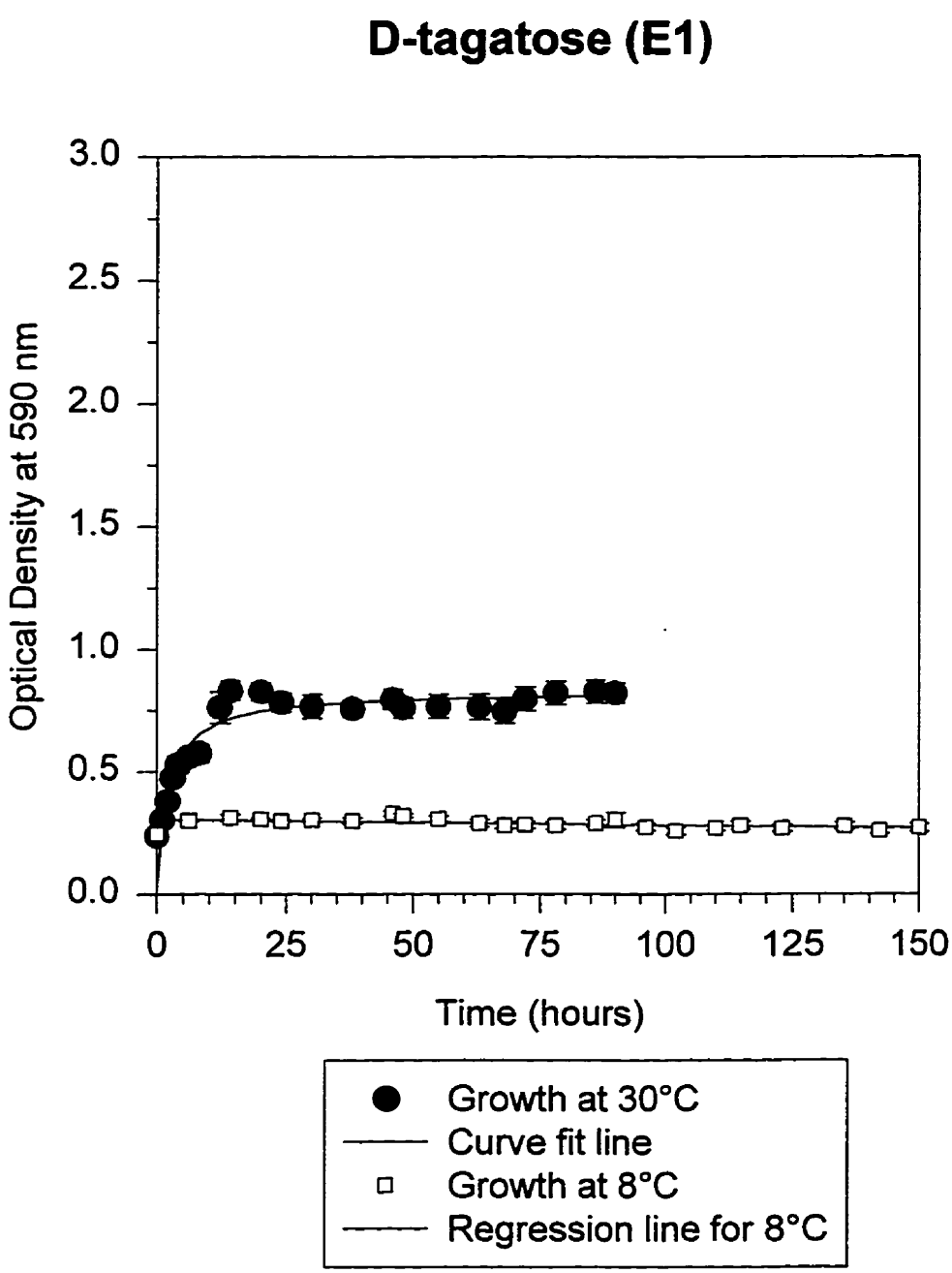

D-psicose (D4)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

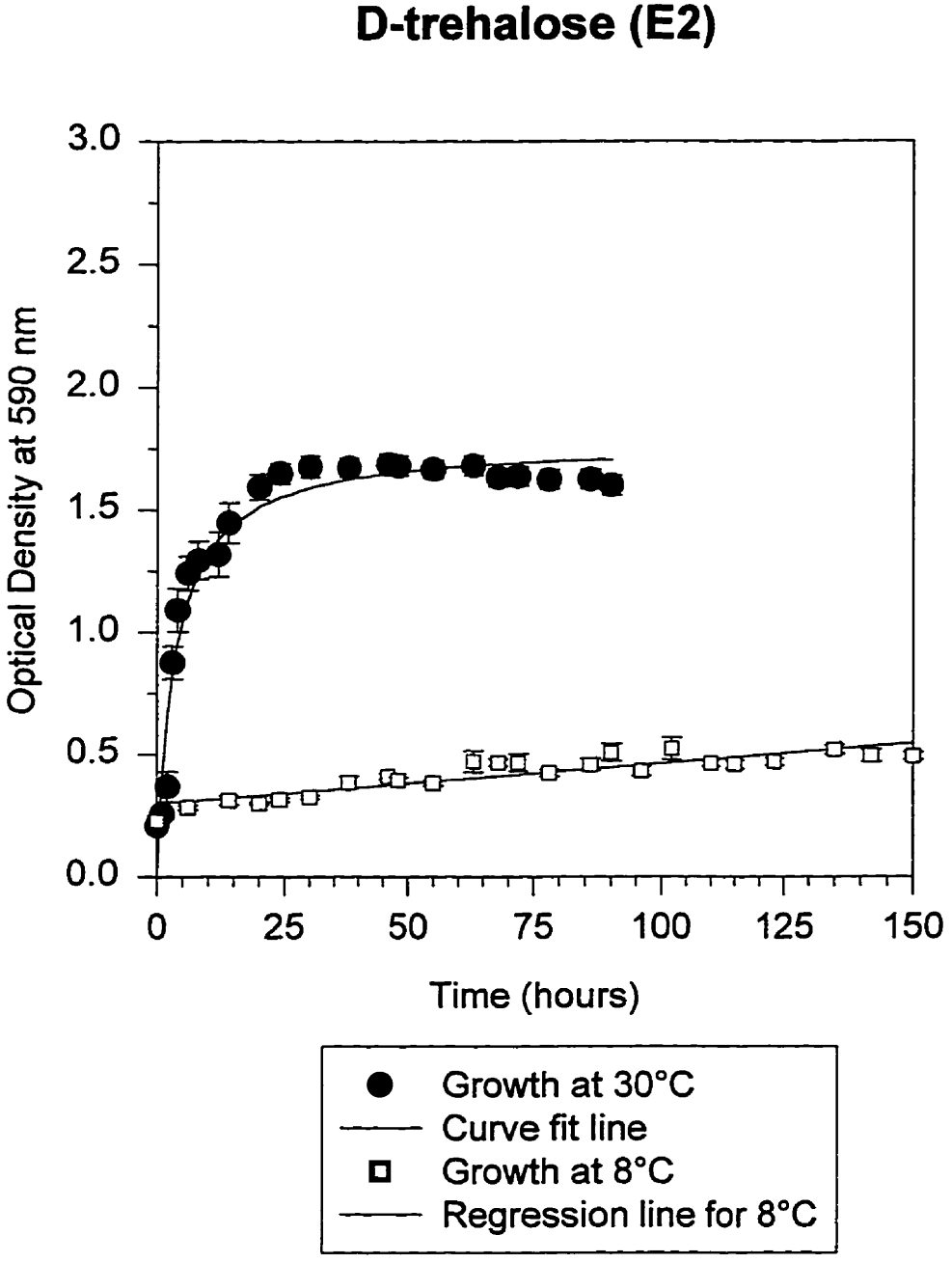

D-ribose (D7)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

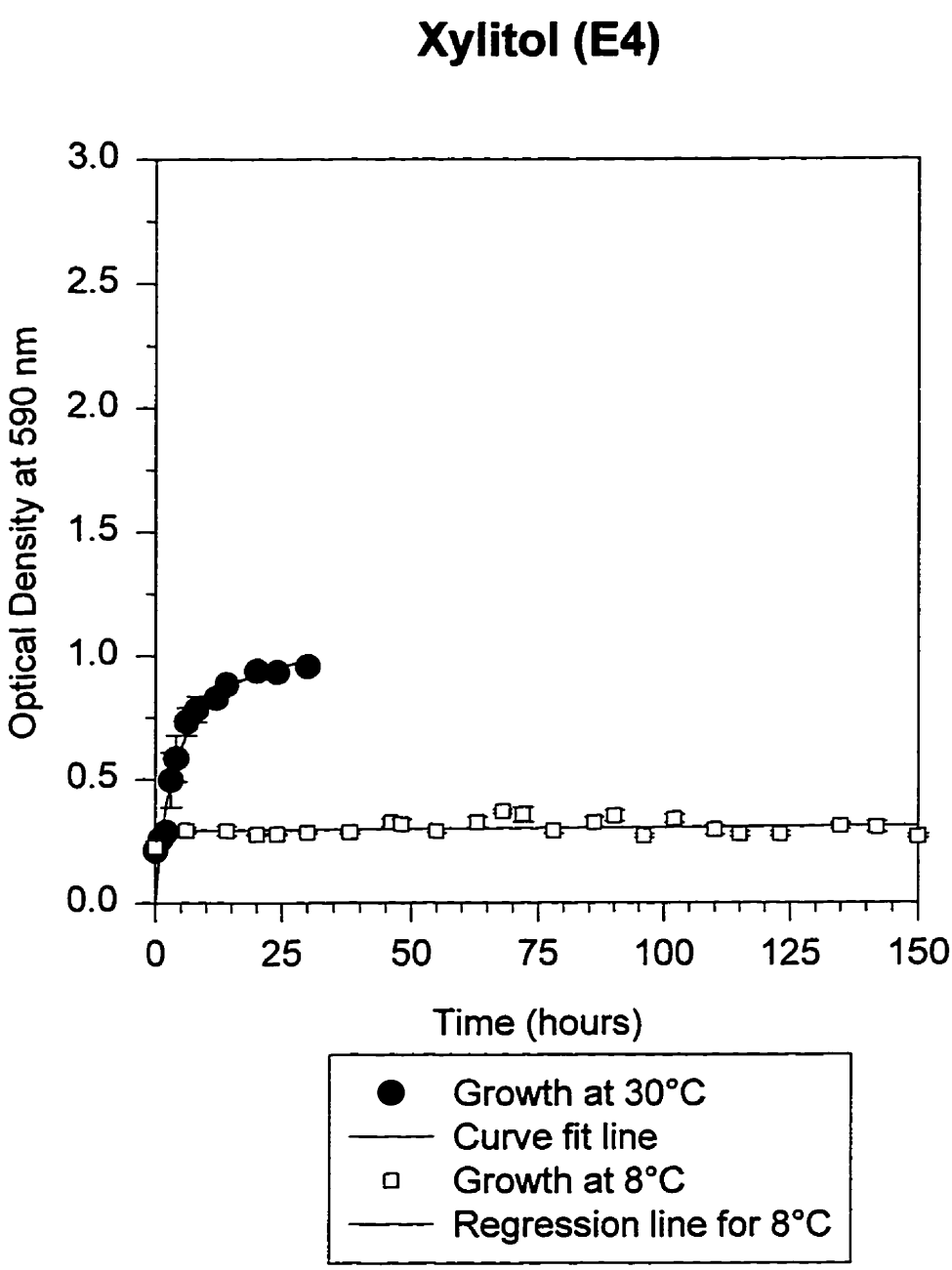
Salicin (D8)

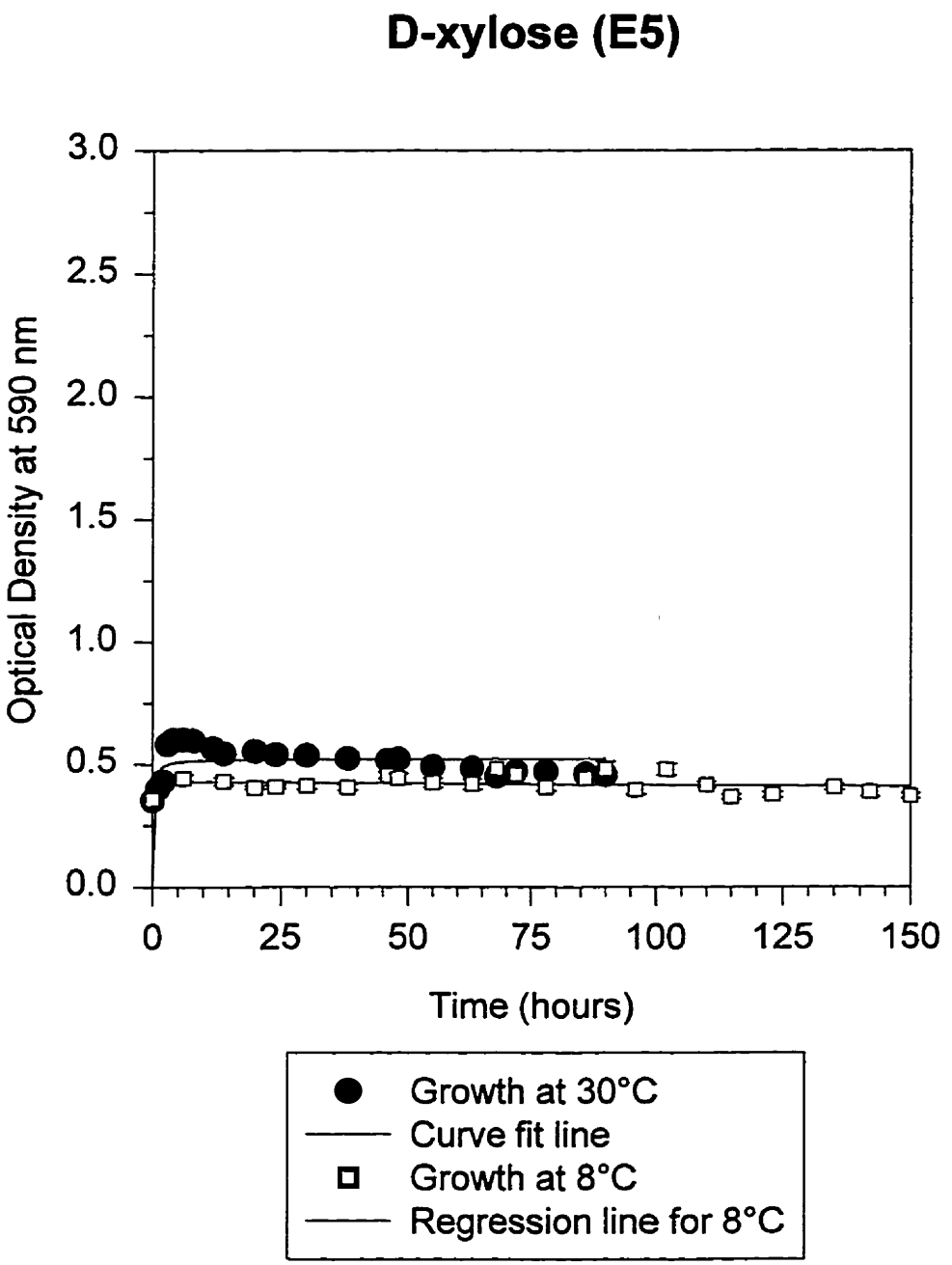

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density at 590 nm
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C


Sucrose (D12)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density at 590 nm
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

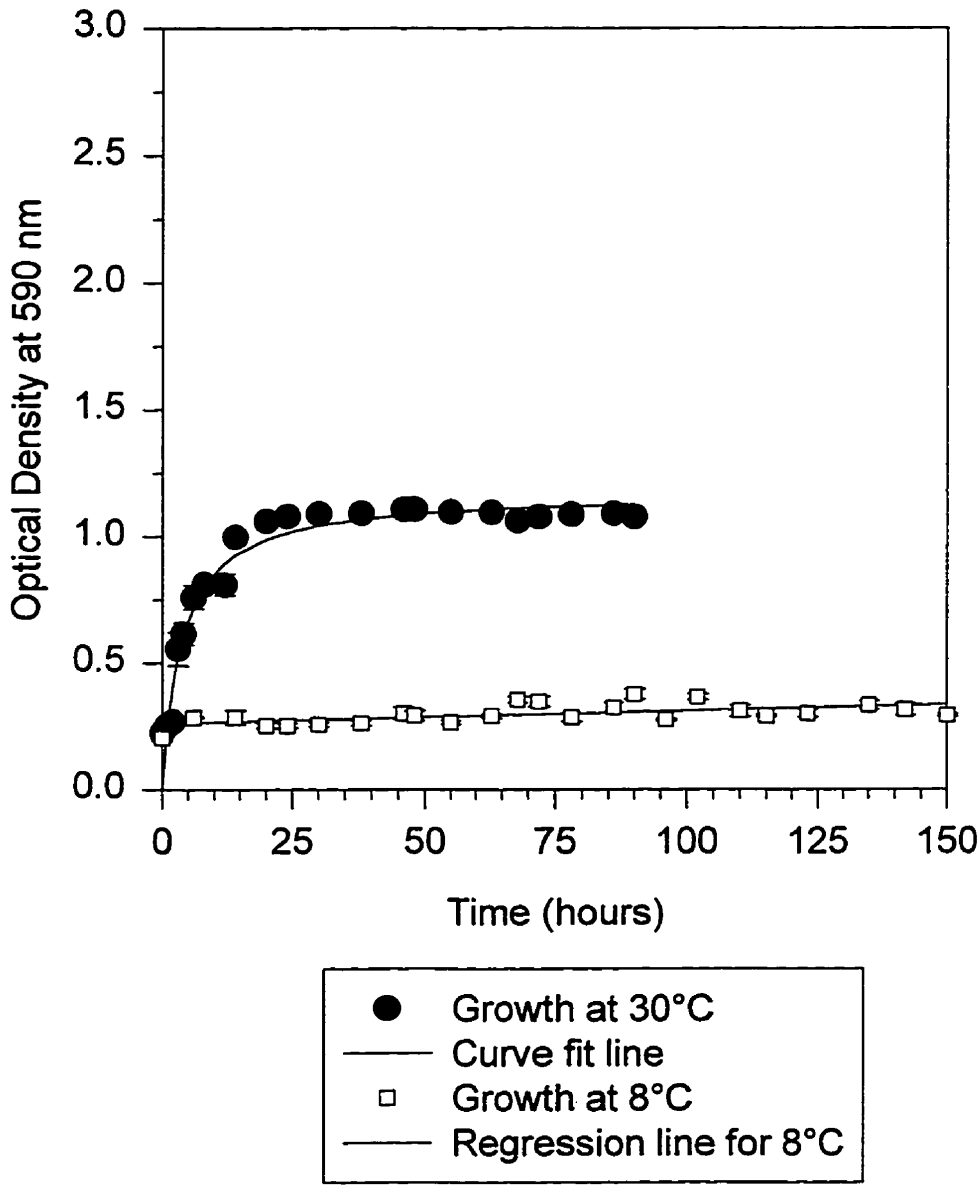

D-tagatose (E1)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

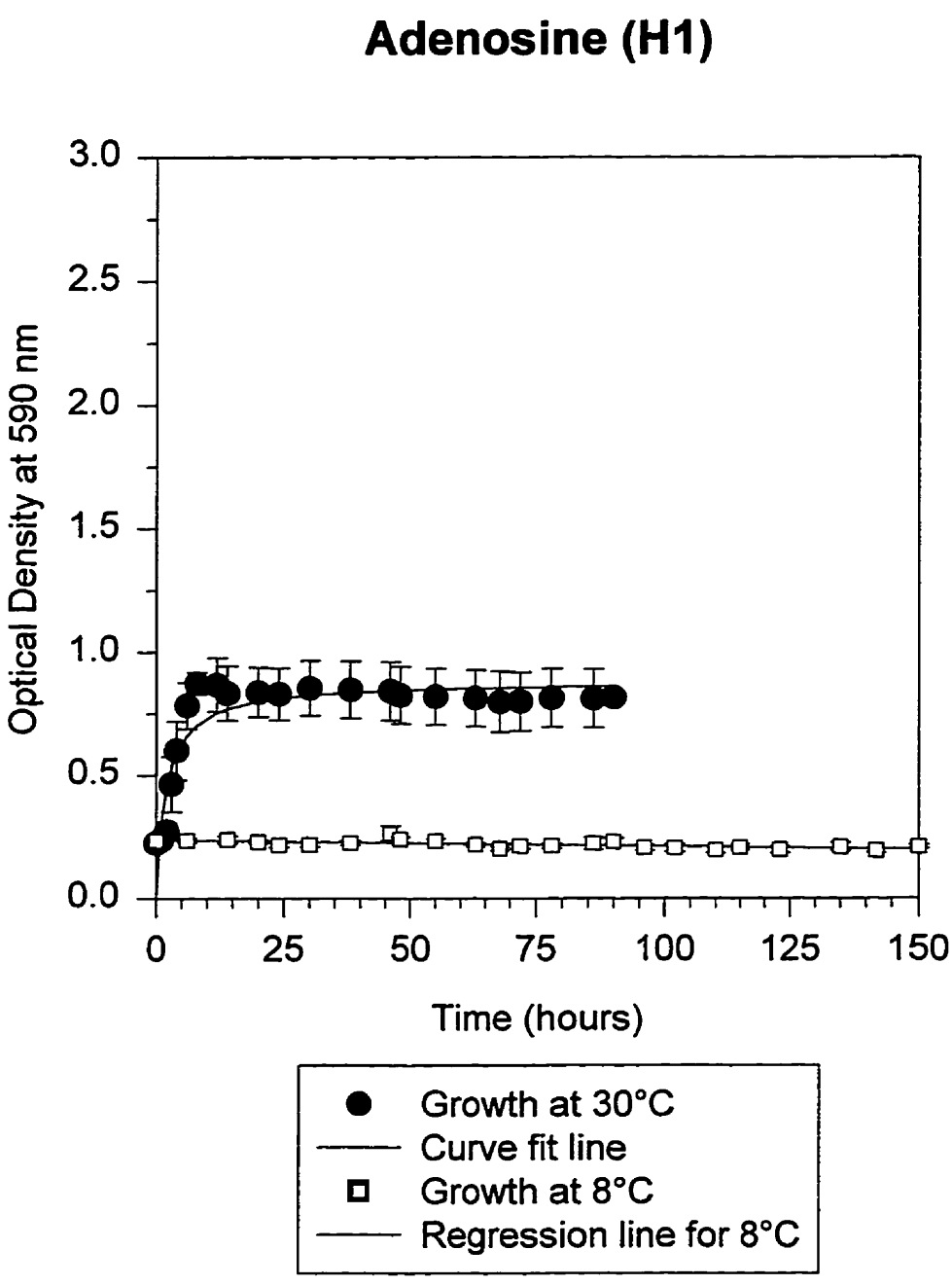

D-trehalose (E2)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

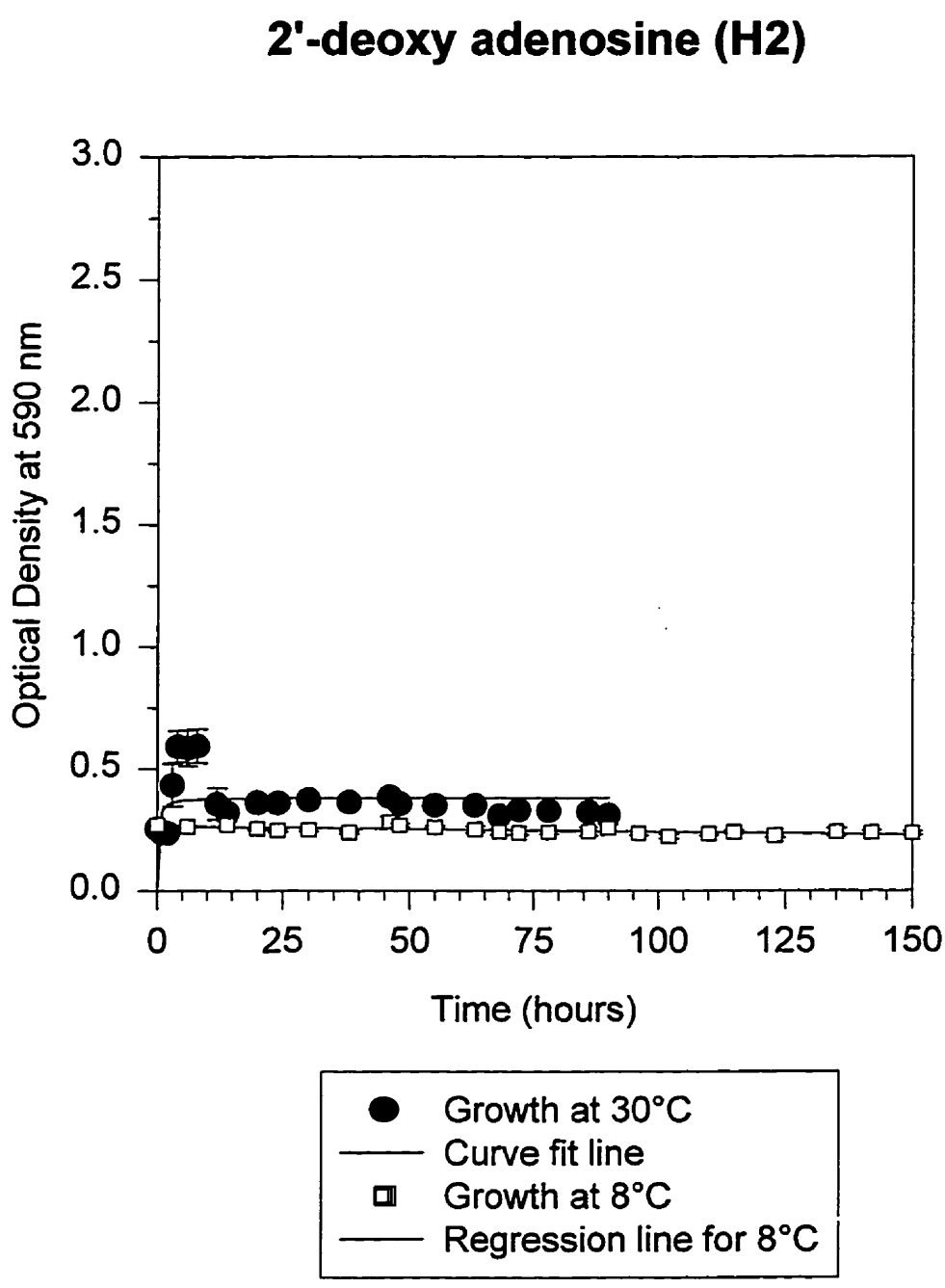

Xylitol (E4)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density at 590 nm
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

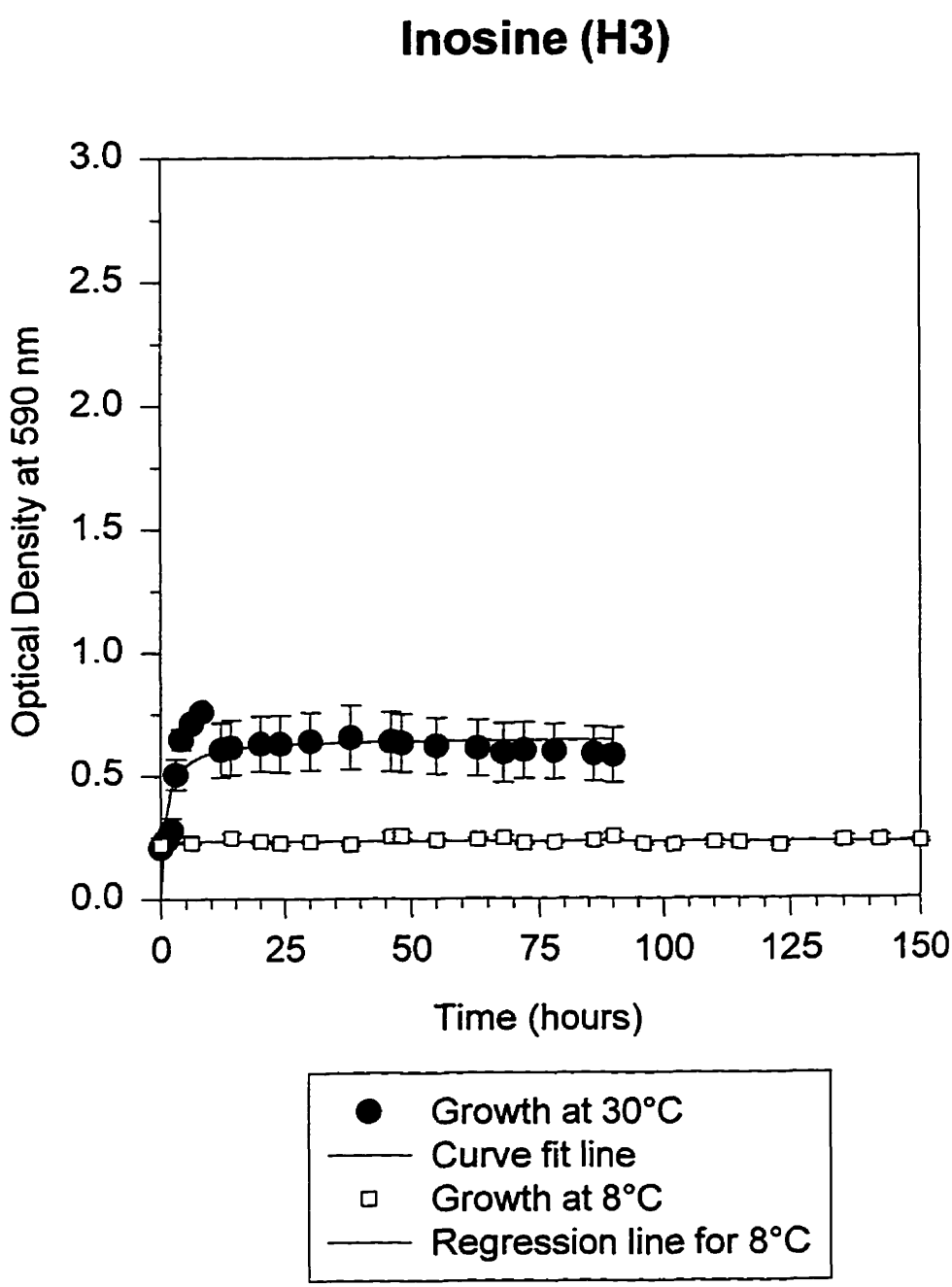

D-xylose (E5)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

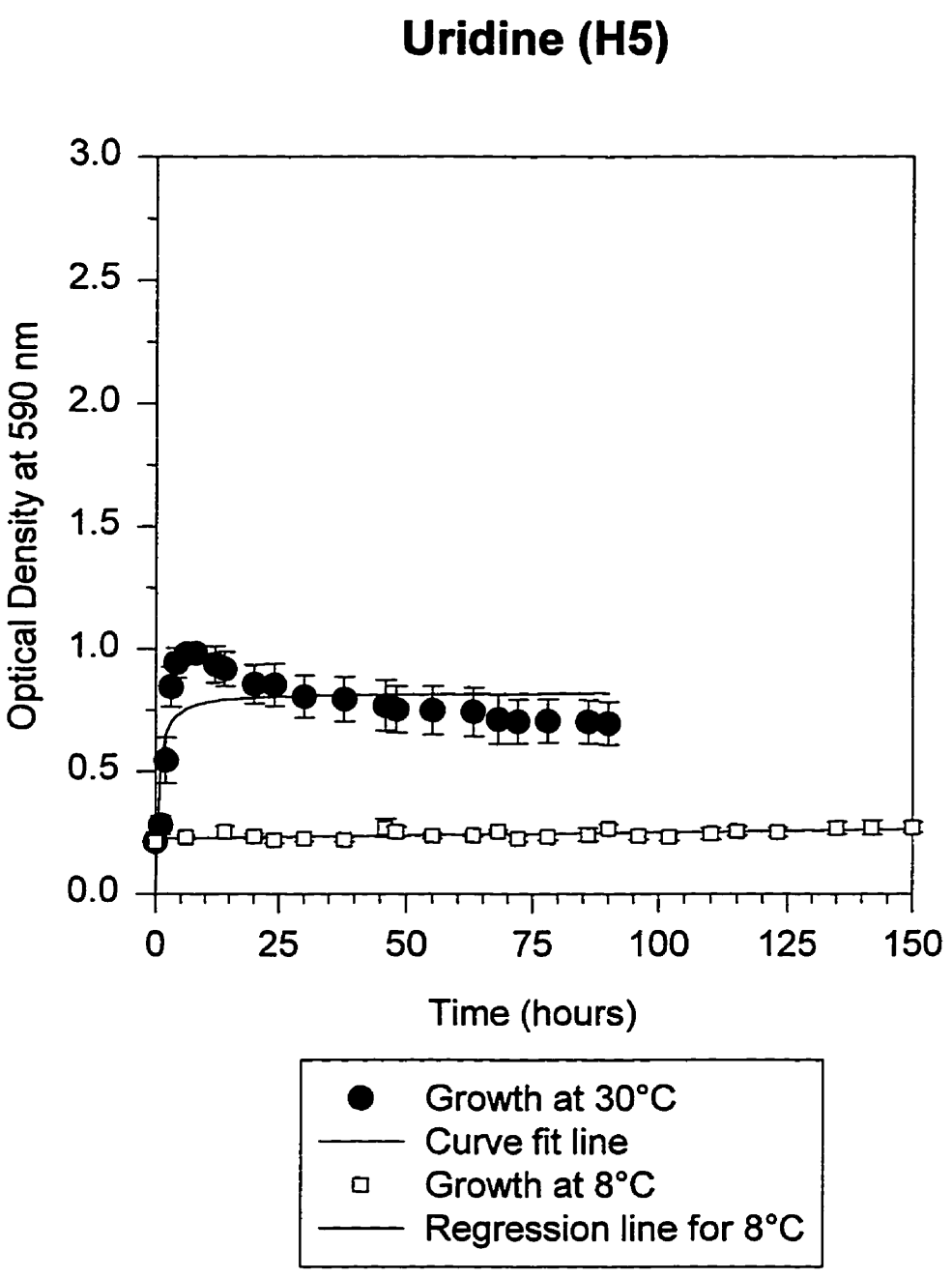
Methyl pyruvate (F6)




Adenosine (H1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Optical Density at 590 nm
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C


2'-deoxy adenosine (H2)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C


Inosine (H3)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C


Uridine (H5)
Optical Density at 590 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
25
50
75
100
125
150
Time (hours)
Growth at 30°C
Curve fit line
Growth at 8°C
Regression line for 8°C

Uridine-5'-monophosphate (H8)