IDENTIFICATION and CHARACTERISTICS of a FUNGAL CONTAMINANT in NAPHTHENIC METALWORKING FLUID

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ABSTRACT

Microbial contamination of metalworking fluids is an ongoing problem. The fluid provides nutrients and thus the microbes degrade the emulsion oil fluid. Samples were obtained from a metal fabrication factory that was experiencing intermittent contamination and fouling of machinery with the resulting biofilm. The fungal contaminant was isolated and then identified by morphological characteristics based on the Saccardo System of Classification. Morphological characteristics were observed with two approaches, light and scanning electron microscopies. The fungal contaminant was identified as *Penicillium*. Culture tubes of naphthenic metalworking fluid were inoculated with a sterile loop of conidia. The fluid was prepared as one part oil to six parts sterile municipal water for incubation periods of 2, 3, 4, and 5 weeks at 23 °C and 37 °C. The interaction between the metalworking fluid and fungal contaminant was characterized by gas chromatography. The gas chromatograph curves were digitized into areas that corresponded to boiling point fractions of standard alkanes. Gas chromatographs showed unique peak differences between culture samples of different ages and temperatures. The boiling point fraction of 60-110 °C contained unique peaks that were observed with *Penicillium* and occurred as early as two weeks of age. The fungal cultures showed decreases in the relative percent of the areas of the gas chromatograph in higher boiling point fractions, by the age of 5 weeks. These fungal chemical byproducts found in the 60-110 °C fraction correspond to compounds of 6-10 carbon atoms. This research demonstrates that *Penicillium* can grow in naphthenic metalworking fluid and thus, alter the chemical composition of that fluid.

INTRODUCTION

Metalworking fluids (MWF), also known as cutting fluid, are base oil/water mixes used in industries. These diverse fluids are used for machining or cutting, metal fabricating, thermal protection or moisture protecting (from air or water) [2]. Metalworking fluids contain base oil and organic additives, which include emulsifiers, corrosion and friction inhibitors, wetting agents, biocides and pH buffers (MWF pH 8.5-9.0). Base oils comprise the bulk of the fluid and are defined as mineral oils (hydrogenated petroleum distillates), synthetic oil (no mineral oil present) or a semi-synthetic (a mix of mineral oil and synthetic). Oil structures can be either naphthenic (one or more cycloalkane groups, but lacking aromatic groups), paraffinic (long, branched alkane groups), or a mixture [2]. Paraffinic oils also contain paraffin wax and are used widely as the base for lubricating oils [16]. Paraffinic oils maintain their properties where higher-temperature applications are needed [2]. Naphthenic oils are used where lower-temperature applications and additives are required [16]. The relative amounts of naphthenic oils are based on the crude oil itself and production processes of those oils. Since MWFs are a mixture, the amount of naphthenic hydrocarbons in a naphthenic MWF may vary from 35% to 40% or 65% to 75%, as reported by Anderson and McHugh [12]. The remaining percentages of hydrocarbons are 50-55% paraffinic and 8-10% aromatic. In addition, the average molecular weight of naphthenic oils used in MWFs is 310, which corresponds to the average carbon numbers of C_{23} [2].

Fungal mats and other biofilms do not appear until the fluid is almost three weeks old. However, the fabricating plant observed cycles of fungal and bacterial counts. This cycling of populations varies depending on which population was first to inhabit the

MWF. Past research has shown that fungi were better able to colonize cutting fluid emulsions that were first colonized by bacteria [13]. The large population change in fungal counts is logical when bacteria are reduced by the use of biocides. This logical conclusion was confirmed by Rossmoore and Holtzman [13]. Their work also showed that if the emulsion was not completely removed, the residual fluid would promote a contaminant population that would be able to compete for resources better. Their study was with *Fusarium*; however, this population cycling can be applied to species of *Penicillium*, which explains the cycling populations observed in the field.

Most manufacturing processes are at lower temperatures, which use naphthenic oils. Naphthenic oil is mixed with water to form an emulsified fluid. These emulsifications are used to coat sheets of metal before the sheet enters the molding presses. The oil prevents the metal from adhering to the press. One of the problems associated with naphthenic oil is that it provides a carbon source for microorganisms, *i.e.*, bacteria and fungi. The population of these organisms can grow exponentially to numbers that negatively affect the final product or interrupt the manufacturing process. The organisms accumulate in the fluid thereby plugging machine filters, which causes down time to replace the filters. The jets that spray the MWF can also become plugged. The brush rollers can become covered with a slim coating of fungal mats and bacteria, necessitating cleaning or replacement of the brush rollers. The accumulation of organisms not only causes production delays through equipment failure, but the organisms themselves can be pressed into the final product. Microbial contamination also deteriorates the fluid by metabolic activities, decreasing the life span of the MWF and prevents proper lubrication.

Life span of MWFs can vary but ranges from eight weeks to six months, but once contaminated MWF life span can be shortened to one week. This results in significant expenditure for fluid replacement and frequent fluid replacement causes considerable loss in production hours for the procedure and downtime for the machinery. Another problem associated with contamination is disposal cost. Costs are high for proper disposal of the contaminated emulsified oil because the oil is considered a hazardous waste. Total expenditures can be significant with metalworking factories needing up to 20 or more presses, each holding 400-500 gallons of emulsion oil. Reduced productivity and increased costs incurred as a result of microbial contamination have spurred research on cause and control (J. Cons, personal communication).

Bushnell [4] noted that Sohngen and Kaserer in 1906 were the first to document microorganisms utilizing hydrocarbons as an energy source. These organisms were pseudomonads and other bacteria. Mineral oils can serve as potential sources of energy and allow bacteria and fungi to colonize the hydrocarbon. Fungi are capable of living in hydrocarbons with the hydrocarbon molecules providing the energy for their growth. Fungi that colonized cutting fluids or other industrial hydrocarbons were not extensively studied until 1961 by Hazzard [9]. The contaminated hydrocarbon source in this study was aviation fuel, which supported 16 different genera of fungi including *Alternaria, Aspergillus, Penicillium, Trichoderma, Cladosporium, Fusarium, Helminthosporium, Ascoidea, Torula, Paecilomyces, Coniothyrium, Curvularia, Epicoccum, Chaetomella, Dicranophora,* and *Bispora*. *Fusarium* was the first documented fungus to show hydrocarbon metabolism of industrial MWFs. The carbon source was *n*-decane and

n-dodecane diesel fuel [6]. The next 10 years of cutting fluid research discussed the chemical control of fungal species in the fluids.

Contamination of MWFs with fungi raises safety concerns. Some fungi produce mycotoxins that have the capacity to pass through the human cellular membrane and disrupt cellular processes. These disruptions may include a simple running nose (rhinitis), or more serious life threatening complications such as allergic reactions and even asthma. Not only do the above respiratory symptoms occur, people can also have chronic sinus problems and eye/vision problems. Long-term exposure with the added effect of high levels of fungal spores may cause severe medical conditions. These conditions may be hemorrhage into the lungs, cancer, hemorrhagic pneumonitis, kidney failure, seizures and hypersensitivity pneumonitis [17]. The age of the person has little effect on fungal infestation. For example, the Centers for Disease Control (CDC) found a link in 1997 between mold contamination and cases of infant pulmonary hemorrhage. At the University of Texas MD Anderson Cancer Center, approximately 15-20% of patients with leukemia die of fungal leukemia caused mostly by *Aspergillus* [17].

Aspergillus species are found everywhere and have been reported as the causative agents of opportunistic infections in man [18]. Opportunistic infections are most prevalent in patients that have suppressed immune systems. *Aspergillus* can infect any organ or organ system including, but not limited to, the nervous system, cardiac system and respiratory system. These organs infected with *Aspergillus* can develop cases of cerebral aspergillosis, meningitis, endocarditis, pulmonary aspergillosis and cutaneous aspergillosis [18]. This genus has also been reported to cause allergic reactions in people who do not have immune deficiencies [19].

Another opportunistic fungus is *Alternaria*. This fungus is also ubiquitous and is a common laboratory contaminant. Infections obviously affect people with immunosuppression, specifically bone marrow transplant patients [20]. Genera of *Alternaria* have been reported to also cause onychomycosis with ulcerated cutaneous (skin) infections and eye infections. On the other hand, healthy individuals may develop hypertrophic sinusitis from *Alternaria* growing in the paranasal sinuses [20].

Cladosporium is another commonly found fungus. This is one of the so-called black molds that is commonly found in wet areas of buildings. It is also readily found in soil. Human infections of *Cladosporium* may be severe when it is introduced into cuts or abrasions. This fungus can cause severe allergies and can be life threatening to people with restrictive airway diseases, *e.g.*, asthma [21]. Chronic *Cladosporium* mycosis can lead to pulmonary emphysema and weaken the immune system [22].

Paecilomyces is another commonly dispersed fungus that is found in soil, dead plants and food products. It has the potential of causing infections in domesticated animals and humans. People may develop corneal ulcers or endophthalmitis from extended wear of contact lenses. *Paecilomyces* is also an opportunistic pathogen of people with immune deficiencies. This genus can also cause allergic disorders [23].

A fairly new opportunistic human pathogen is the fungus *Curvularia*. This pathogen infects people who have immune system deficiencies. The unique aspect of *Curvularia* is that it has the potential to cause phaeohyphomycosis, allergic sinusitis, allergic bronchopulmonary disease or endocarditis in patients with intact immune systems [24].

Fusarium is ubiquitous and an opportunistic human pathogen. The species of *Fusarium* are well known plant pathogens. However, they can cause superficial and systemic infections in humans. Opportunistic infections occur in immunosuppressed people, including but not limited to, transplant patients. *Fusarium* species have caused keratitis, endophthalmitis, sinusitis, pulmonary infections and endocarditis. Not only are the above diseases problematic, but also the mycotoxins produced by *Fusarium* are of concern. The toxins may cause allergic reactions or be carcinogenic over long-term consumption of contaminated grain products [25].

The present study started as an investigation of the microbial contaminants (both bacterial and fungal) at a General Motors Fabricating Plant (2238 West Bristol Road, Flint, Michigan 48553). This plant uses Naphthenic Oil Draw #59 (Quaker Chemical Company) as the stock oil in a 1:7 dilution (v:v) with unfiltered city tap water. This emulsion lubricant is stored in tanks at temperatures of 32-38 °C in the basement of the facility. When the molding presses are in operation, the lubricant is pumped to the first floor presses, sprayed onto the sheet metal which moves on brush roller conveyors to the metal press for forming. The emulsion lubricant allows for the separation of the newly formed part, (*e.g.,* automobile engine hood, door panel, etc.) from the molding press. Excess fluid is collected and recycled back to the storage tanks. Time is needed to replace parts or fluid and the added cost of $10,000s worth of biocides to control the microbial populations. The fabricating plant was experiencing incidences of random contamination because the available biocide treatment was ineffective. Previous attempts to identify the microbial agent by General Motors had proven unsuccessful. Past research at the facility has not dealt with fungi that contaminate naphthenic MWF. Therefore, the

present investigation will identify the microbial agent(s) and show that the contaminant(s) is capable of altering the composition of the naphthenic metalworking fluid. Although, fungi may cause illness, they are natural recyclers that can degrade complex hydrocarbons thus were a likely cause of contamination.

MATERIALS and METHODS

Sampling of Contaminated MWF

The factory operating temperature of the fluid averaged 37 °C with ambient humidity. Ten random surface samples were collected with sterile swabs from five tank systems, floors and walls around the MWF storage tanks, and two oscillating air fans. The swabs were placed into sterile plastic bags for transport back to the lab. Swab samples were plated and then stored at 4 °C.

Isolation of Fungal Contaminant

Inoculated plates of Sabouraud Dextrose Agar (SDA, Difco Laboratories; Sparks, MD) were incubated at 37 °C and at room temperature for 4 days or until hyphal mats were about 2 centimeters in diameter (4-7 days). No bacterial growth was observed on any plates.

For production of spores, mycelial samples were removed from SDA via flamed sterilized scalpel and plated onto potato dextrose yeast agar (PDYA, Difco). Plates were prepared as described in *CRC Press Handbook of Microbiological Media.* Cultures were incubated two days at 37 °C. PDYA was used to induce sporulation.

Identification of Fungal Contaminant

Reproductive cell samples were collected from PDYA and observed. These morphological characteristics of hyphae were visualized by using the "tape transfer method." This method is an inexpensive way to collect hyphal samples. A piece of clear plastic tape approximately seven centimeters long was gently pressed against the fungal mat. The tape is then placed with hyphae sample against a glass slide and observed under

the microscope at a magnification of 400x. For taxonomic identification, the Saccardo System of Classification was used [3]. This is a morphological system of the reproductive structures, *i.e.*, conidiophores and conidia.

Electron Microscopy

PDYA samples approximately five millimeters square were used for Scanning Electron Microscopy (SEM). First, the specimen was dried for four hours in an incubator at 37 °C. The mounting stub was coated with carbon paint (Aquadag Colloidal Graphite by Ted Pella, Inc., Redding, CA) and the specimen was positioned on the stub while the paint was wet. The paint was allowed to dry for ten minutes. The LVC-76 Sputter Coater (Plasma Sciences, Inc; Lorton, VA) was used to gold sputter the specimen as described below. The chamber cylinder was removed and the sample stub was placed in the holder. Then the chamber cylinder was replaced and seated firmly in the grooved base. Next the 3-way selector valve was set to "Vacuum." The machine was set to "Coat" and the power was turned on. The chamber pressure was allowed to reach 75 mT (millitor; never below 50 mT) and the Argon gas was turned on by opening the gas tank. Next, the 3-way selector valve was set to "Gas" to allow the Argon into the chamber, and at this point the chamber pressure returned to about 250 mT. The pressure was brought back near 150 mT and the timer was set for nine minutes. Then the "Process Button" was depressed and released. Plasma glow and the vacuum pressure were observed. This glow should appear as a flat-plate-like cloud (not a bubble) in order to ensure an even coating of the specimen. If necessary, adjustments can be made with the needle valve (on top) to flatten the cloud and keep the vacuum pressure near 150 mT. After the timer stopped, the 3-way selector valve was set to "Vacuum" and the Argon gas tank and

power were turned off. Then the 3-way selector valve was set to "Vent" for five seconds, and then reset to "Vacuum." Next, the chamber cylinder was removed (straight up) and the sample stub was removed. Finally, the chamber cylinder was repositioned.

The sputtered sample stub was placed into the scanning electron microscope. An AMRAY 1810D Scanning Electron Microscope: Diffusion Pumped SEMS without an Ion Pump (Amray, Inc.; Befford, MA) was used for the microscopy. Photographs were taken at magnifications of 398x to 1370x with a Mitsubishi P4OU Video copy Processor (Figures 1 and 3). The above electron microscopy procedures were used to aid in the identification of the fungal sample. The procedures are specific to the equipment used for the investigation.

Contaminant Usage of MWF

For cultivation of the fungal contaminant, MWF was prepared using Quaker stock oil Draw #59 diluted 1:7 with sterile tap water (1 part oil to 6 parts municipal water supply, Flint, MI). The stock oil chemical composition was ~65% naphthenic oil (Appendix 1: Table A). Seven milliliters of the emulsion oil mix was aseptically pipetted into sterile 25mm x 150mm glass, screw-cap culture tubes. Sterile loop transfers of hyphae/spores were placed into the experiment tubes. Control and experimental tubes were prepared as described above, for an extended incubation of 2 weeks, 3 weeks, 4 weeks and 5 weeks with incubation at 37 °C and room temperature ~23 °C (total of 16 tubes). These tubes were agitated at 4 rpm in a New Brunswick Gyrotory Water Bath Shaker Model B76 (New Brunswick, NJ) at 37 °C and the 23 °C tubes were placed on an insulated foam sheet on top of the shaker.

Gas Chromatography

One milliliter of each tube of MWF containing the inoculum as well as each control tube was placed into a microcentrifuge tube and spun at 15,000 rpm for 15 minutes with an Eppendorf 5412 centrifuge (Brinkmann Instruments; Westbury, NY). The supernatant was removed and placed into a new microcentifuge tube. The sample was vortexed and approximately a 0.5 mL of diethyl ether was added to the supernatant and vortexed. This ether layer was then removed via micropipette and placed into a small, brown screw-cap bottle, wrapped with Parafilm, and stored at 5 °C for Gas Chromatograph (GC) analysis. GC analysis used 0.5 μL (Hamilton 7000 Series Microliter Syringe; Reno, NV) of ether as the sample size. Analysis was performed on a Shimadzu GC-14A (Kyoto, Japan) with a flame ionization detector (FID) and split/splitless injector coupled with an Integrator/printer CR-501 (Shimadzu; Kyoto, Japan). The GC was fitted with an Rtx-5 crossband capillary column (5% diphenyl-95% dimethyl polysiloxar, 30 meter, 0.25 mm ID, 0.25μm). Helium, nitrogen, air and hydrogen were provided by AGA Gas: Cleveland, OH. The range on the GC was set at 10^0. The other analysis parameters of the CR-501 were defaults of the machine as follows. The width was set at 5 seconds, the slope at 70 μv/min, the drift at 0 μv/min, minimum peak area at $1x10^6$, the time when width doubles (T.DBL) set at 0 minutes, attenuation at 2, stop time was 45 minutes and the speed of the chart was 10 mm/min. The injector temperature was 150 °C and the detector temperature was 300 °C. The temperature was initially ramped from 50 °C to 150 °C at a rate of 10 °C/minute. The temperature was then ramped a second time at a rate of 5 °C/minute to a final temperature of 280 °C where the temperature was held for 10 minutes. The resulting chromatograph

was integrated into time range zones using Un-Scan-It software (Silk Scientific; Orem, Utah). The zone boundaries were determined by the retention times of standard (C_8 to C_{40} standards, Fluka Chemie AG, St. Gallen, Switzerland) alkanes. The zones were then defined by the retention times of those alkanes and grouped into areas relative to the number of carbons. The peak size and location was also noted. The area of each zone is reported as a relative percent of the total area of the gas chromatograph. The areas were then compared between the control sample and the test sample. Between each GC sample 1 μL of diethyl ether was injected into the GC to clean the column by running the temperature program for 10-14 minutes or multiple 1 μL diethyl ether injections until no peaks appeared in the first 10 minutes of the temperature program. The above temperature program was designed for the equipment used as modified from Anderson's procedures [2]. The GC data were used to identify notable relative changes in the chemical composition of the naphthenic metalworking fluid. Appendix 1: Table B provides the boiling points of some selected hydrocarbons that may be found in naphthenic MWFs.

RESULTS

Identification of Fungal Contaminant

The plated samples only produced one kind of fungus. There was no bacterial contamination or other fungal contamination on any of the SDA or PDYA plates. The PDYA fungal mat was white in color, but as the sample matured it turned tan. The "tape transfer method" and SEM revealed septate hyaline hyphae with phialidic conidiophores arranged as a brush (Fig. 1 and 2). The chains of conidia were elongated and basipetal (Fig. 3). The culture isolated from the original factory sample and the metalworking fluid sample were consistent in appearance and growth rate on PDYA as well as in light and electron microscopic analyses. Also, the contaminant's morphology was the same at both temperatures (room and 37 °C). Periodically the controls were plated and produced no bacterial or fungal growth.

The fungal contaminant had simple septate hyphae, which classified it as Subphylum Ascomycotina. The isolate was classified as Family *Moniliaceae* because the conidiophores are single or in loose clusters and the conidia and conidiophores are hyaline (light in color). Conidia observed were one-celled and globose to several times longer than wide. Conidiophores were simple, separate, and branched with phialides present in upright clusters, brush-like arrangement. Based on the Saccardo System taxonomically identified the contaminant as *Penicillium*. The phialides are visible in both yellow and blue light (microscope: Microstar IV model 410, Cambridge Instruments; Buffalo, NY), see Figures 2 and 4. Identification of the species has been inconclusive (Appendix 2).

Gas Chromatography

The control was uncontaminated MWF that was incubated at the same temperature for the same time length as the test group. The test samples were MWF contaminated with *Penicillium* spores. The test sample incubated at factory operating temperature (37 °C) for two weeks showed numerous detailed peaks in the retention time range of 18-22 minutes (Fig. 5). These peaks indicate many compounds being present. Peaks near the 4-minute mark are smaller when compared to the control (Fig. 6). These peaks were in the time zone of 2-4 minutes. This time zone corresponds to the boiling points (bp) of 6-10 carbon atoms. The control had a percentage of 0.43, whereas the test's percentage was 0.32 (Table 1). Time zone 4-12 minutes (bp 90-160 °C) showed a minor difference in the relative area of the control (13.96) to the test (11.94). A minor difference was also seen in the time zone 12-20 minutes and 20-28 minutes (Table 1). The percentage values for the contaminant's retention time zones 20-28 minutes and 28-36 minutes showed an increase from zone to zone by 33.8% and 21.4%, respectively (Table 1).

Table 1: Two-Week-Old Sample at 37 °C

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.43	0.32
10-14	4-12	90-160	13.96	11.94
14-19	12-20	160-200	5.21	5.58
19-25	20-28	200-240	6.48	8.67
25-30	28-36	240-280	10.47	12.71

Values for the Control and Test are relative percents of the total area.

The two-week-old test sample at room temperature showed an extensive number of compounds being present in the MWF (Fig. 7). The contaminant doubled the area in the retention time zone of 2-4 minutes. The relative area of the control was 0.33%

(Fig. 8), whereas the contaminant's percentage was 0.75 (Table 2). Noticeable changes with the contaminant's chromatogram are in the zones of 4-12 minutes, 12-20 minutes, and 20-28 minutes. The contaminant's percentage for the zone of 4-12 minutes showed a 62% increase when compared to the control (7.28 to 11.82, Table 2). The zone of 12-20 minutes (bp 160-200 °C) showed many peaks on the chromatogram, but had a similar area (test = 6.13%; control = 4.5%). The noticeable changes in the room temperature, two-week-old sample were also in the retention time of 20-28 minutes (Fig. 7 and Table 2). This change in the area under the curve from the control percentage when compared to the contaminant percentage was twice the area.

Table 2: Two-Week-Old Sample at Room Temperature

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.33	0.75
10-14	4-12	90-160	7.28	11.82
14-19	12-20	160-200	4.5	6.13
19-25	20-28	200-240	4.88	9.81
25-30	28-36	240-280	11.61	13.24

Values for the Control and Test are relative percents of the total area.

The three-week-old test sample at 37 °C had similar areas compared to the control (Fig. 9). The relative percentage of the area in the time zone of 2-4 minutes for the control was 0.26, whereas the test percentage was 0.55 (Fig. 10 and Table 3). The time zone of 4-12 minutes, which corresponds to 10-14 carbon atoms, showed the control had a percentage of 7.39. The test showed a decrease in its percentage of the same area (7.27, Table 3). For the gas chromatograph time ranges of 12-20 minutes and 20-28 minutes, the control values are 10% less than the contaminant values (Table 3). Peaks at minutes 4 and 11 were detected in the control. Peaks at minutes 18, 21, and 23 were not observed in the presence of the fungal contaminant. A notable change to the MWF was

with the peak at 14 minutes, which was only slightly visible in the presence of *Penicillium*, whereas in the control it is almost double.

Table 3: Three-Week-Old Sample at 37 °C

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.26	0.55
10-14	4-12	90-160	7.39	7.27
14-19	12-20	160-200	3.10	3.32
19-25	20-28	200-240	3.43	3.84
25-30	28-36	240-280	13.37	14.51

Values for the Control and Test are relative percents of the total area.

The three-week-old culture incubated at room temperature also produced notable changes within some time ranges (Fig. 11). The zones of 2-4 minutes and 4-12 minutes showed a limited number of peaks both in the test and the control chromatographs (Fig. 11 and Fig. 12). These areas had similar percentages (Table 4). Retention time ranges of 12-20, 20-28, and 28-36 minutes produced the following percentage values. The control's percentage for 12-20 minutes was 4.55 (Fig. 12), whereas the test's percentage was decreased by 25% (3.37, Table 4). The test's percentage for 20-28 minutes was increased to 3.84 and the control was 3.04. For the time range 28-36 minutes, which corresponds to 25-30 carbon atoms, the presence of *Penicillium* increased the relative percentage of that zone when compared to the control (Table 4). The peak at the retention time of 9.75 minutes was present in the contaminated fluid, but not the control. In addition, the peak corresponding to the compound identified at the retention time of 10 minutes was about three times larger in the presence of the fungal contaminant. Peaks that are not produced in the presence of the fungal contaminant would be peaks at 20, 21, and 28 minutes.

Table 4: Three-Week-Old Sample at Room Temperature

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.23	0.28
10-14	4-12	90-160	6.49	6.08
14-19	12-20	160-200	4.55	3.37
19-25	20-28	200-240	3.04	3.84
25-30	28-36	240-280	11.53	16.29

Values for the Control and Test are relative percents of the total area.

The four-week-old test sample at 37 °C showed only slight differences in the gas chromatograph compared to the three-week-old gas chromatograph. The 2-4 minute zone of the control had a percentage of 0.18, whereas the contaminant had a percentage of 0.07 (Table 5). A decrease was also observed in the time range of 4-12 minutes. The control percentage for this area was 10.87, compared to the contaminant sample percentage of 8.92. The time range of 12-20 minutes showed that the area of the test doubled compared to the control (test = 6.07; control = 3.37). Another time range that had similar relative areas was the 20-28 minutes. The control was 2.23%, whereas the test was 2.54%. The most noticeable changes between the control and the contaminant are peaks in the time ranges of 10-12 minutes and 22-24 minutes. Contaminant peaks are smaller in the 10-12 minutes range. In the time range of 22-24 minutes, the contaminant produced distinct peaks. In addition, the contaminant produced smaller peaks when compared to the control, except for peak 23, which is larger in the contaminant (for compounds present in both the control and contaminant) (Fig. 13 and Fig. 14).

Table 5: Four-Week-Old Sample at 37 °C

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.18	0.07
10-14	4-12	90-160	10.87	8.92
14-19	12-20	160-200	3.37	6.07
19-25	20-28	200-240	2.23	2.54
25-30	28-36	240-280	25.14	20.89

Values for the Control and Test are relative percents of the total area.

The contaminant MWF culture incubated at room temperature for four weeks showed slight changes compared to control when analyzed by gas chromatography (Fig. 15). Time range of 2-4 minutes showed slight peaks in both the control and the test samples. These relative percents varied only by 0.01%. Time range of 4-12 minutes for the control had a percentage of 11.95 compared to the test's percentage of 9.9 (Table 6). A decrease from the control to the test was also observed in the time zone 12-20 minutes. The control's percentage was 5.17 compared to the test's percentage of 2.98 (a decrease of 42%) (Table 6). The presence of *Penicillium* produced the compound that resulted in the peak at 20 minutes, which is not present in the control (Fig. 16). The *Penicillium* has decreased the relative area in the zone 20-28 minutes (test = 3.22; control = 7.43).

Table 6: Four-Week-Old Sample at Room Temperature

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.19	0.18
10-14	4-12	90-160	11.95	9.9
14-19	12-20	160-200	5.17	2.98
19-25	20-28	200-240	7.43	3.22
25-30	28-36	240-280	23.6	16.33

Values for the Control and Test are relative percents of the total area.

The five-week-old MWF culture incubated at 37 °C produced similar peaks in comparison to the control (Fig. 17 and Fig. 18). Some peaks changed only slightly whereas others showed greater changes. These changes occurred (when

compared to control) as peaks or multiple peaks being present. The control time range of 2-4 minutes had a percentage of 0.25, whereas the test culture's percentage was 0.52 (Table 7). Control time range of 4-12 minutes had a percentage of 10.44 compared to the test's percentage of 7.68, which is a decrease of 26%. Time ranges 12-20 and 20-28 showed a decrease in the area for the test sample. However, for time range 28-36 minutes, the control's percentage was 23.99 compared to the test percentage of 15.3 (a 36% decrease) (Table 7). Fungal presence produced peaks at 3 and 4 minutes. The composition of the MWF also changed in the peak regions 10-11 minutes and 12-14 minutes. The fungal sample produced more peaks in those regions. The contaminant *Penicillium* in five-week-old naphthenic MWF at 37 °C produced an extra peak at 23 minutes. Also, by the fifth week the relative amount of individual compounds that were being produced or chemically altered by the *Penicillium* have decreased at factory operating temperature.

Table 7: Five-Week-Old Sample at 37 °C

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.25	0.52
10-14	4-12	90-160	10.44	7.68
14-19	12-20	160-200	4.5	4.09
19-25	20-28	200-240	5.99	5.5
25-30	28-36	240-280	23.99	15.3

Values for the Control and Test are relative percents of the total area.

The sample inoculated at room temperature and at five weeks old produced the following changes (Fig. 19 and Fig. 20): time range 2-4 minutes showed a decrease in the test area having a percentage of 0.55 compared to the control's percentage of 0.69 (Table 8). Another change was observed in the area at the time range of 4-12 minutes. This increase was about 36% with the control percentage being 14.14 and the

test percentage was 19.26 (Table 8). Time range 12-20 minutes also showed an increase in the relative percentage of that area to the total area. This increase was approximately 51% from the control (5.89) to the test (8.94, Table 8). The time range of 20-28 minutes at five weeks old and at room temperature also showed a decrease in the area percentage. The control percentage was 7.04 compared to the fungal test percentage of 4.71, a decrease of 33%. In general, at room temperature the culture produced smaller peaks. The compound that formed peak 3 was almost depleted.

Table 8: Five-Week-Old Sample at Room Temperature

# of Carbon Atoms	*Minute*	*°C*	*Control*	*Test*
6-10	2-4	70-90	0.69	0.55
10-14	4-12	90-160	14.14	19.26
14-19	12-20	160-200	5.89	8.94
19-25	20-28	200-240	7.04	4.71
25-30	28-36	240-280	13.82	15.48

Values for the Control and Test are relative percents of the total area.

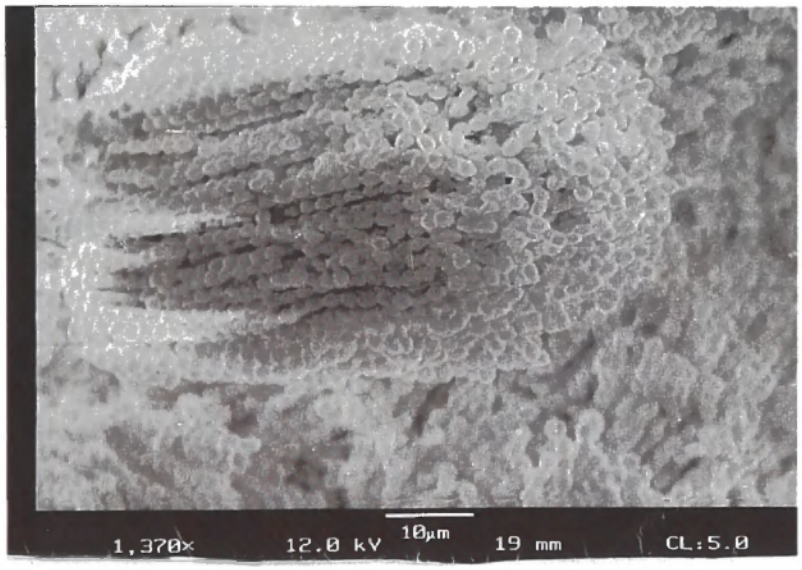

Fig. 1: Septate hyaline hyphae with phialidic conidiophore, arranged as a brush.

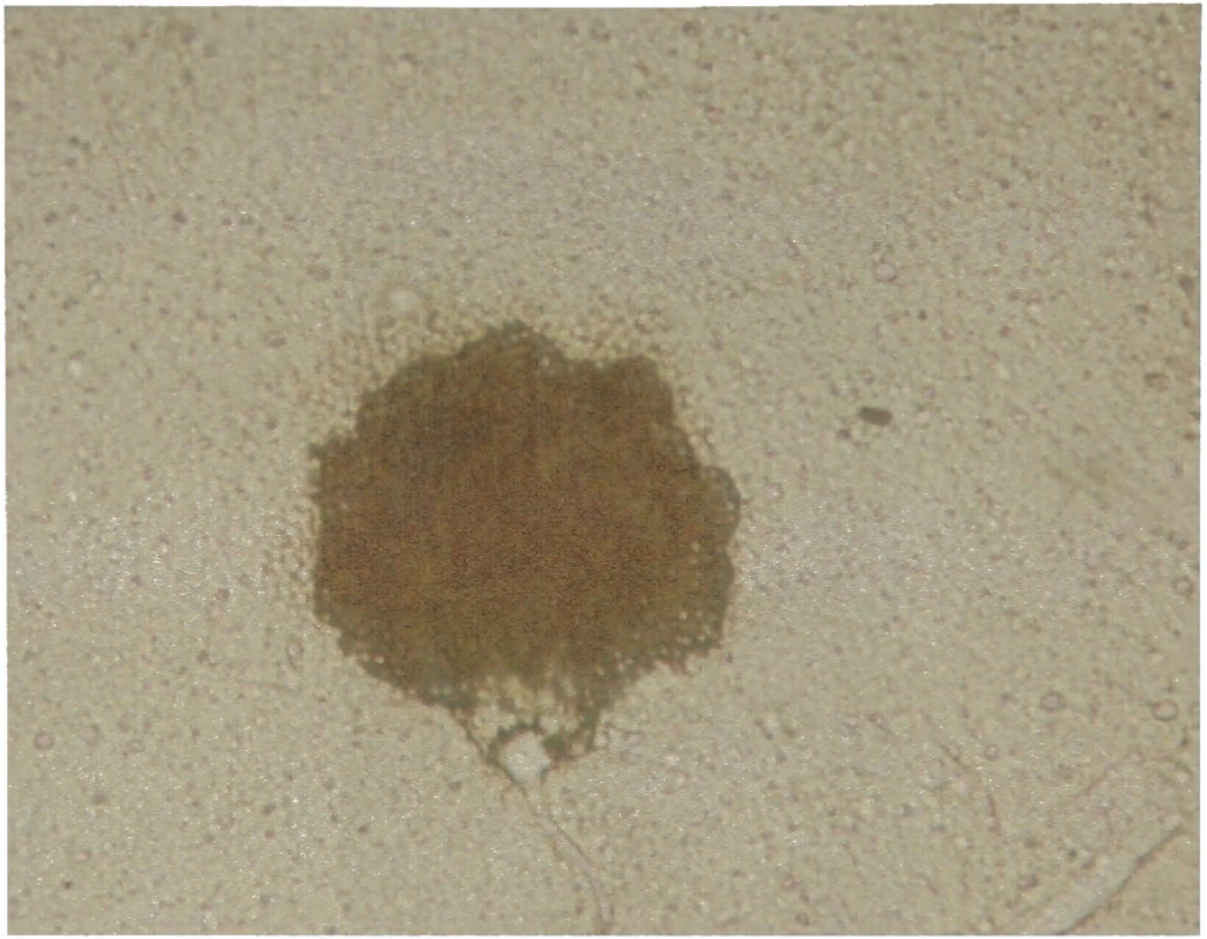

Fig. 2: Phialides and stalks support conidial chains.
Phialides visible in yellow light.

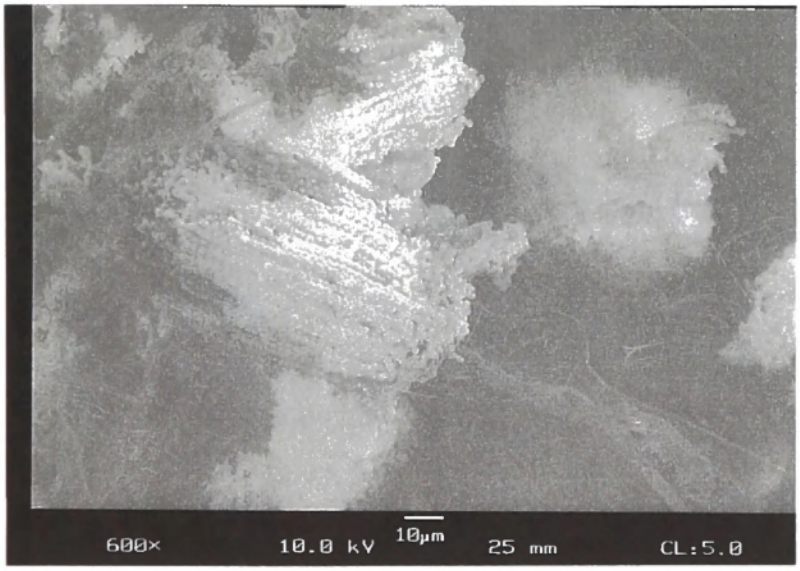

Fig. 3: Chains of conidia were elongated and basipetal.

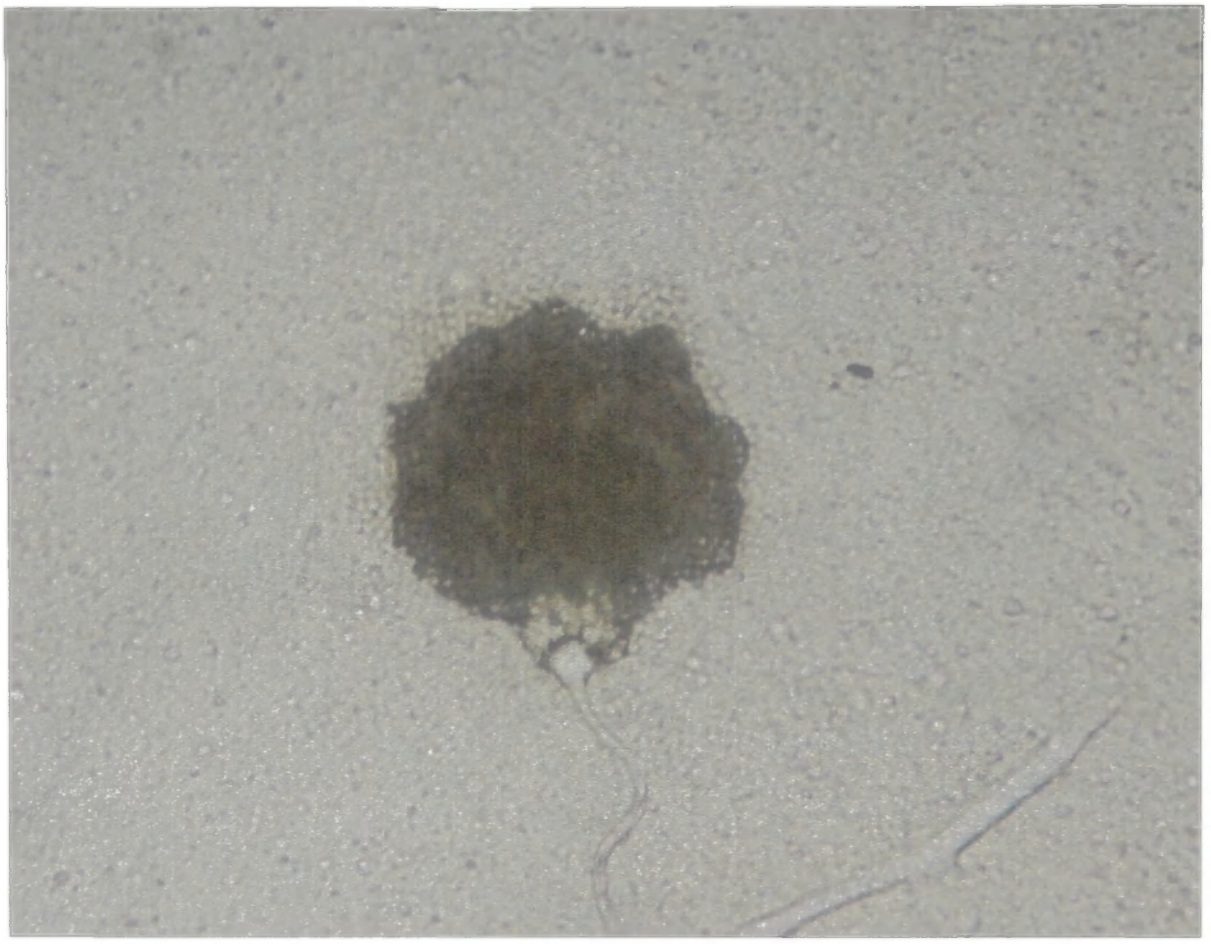

Fig. 4: Phialides visible in blue light.

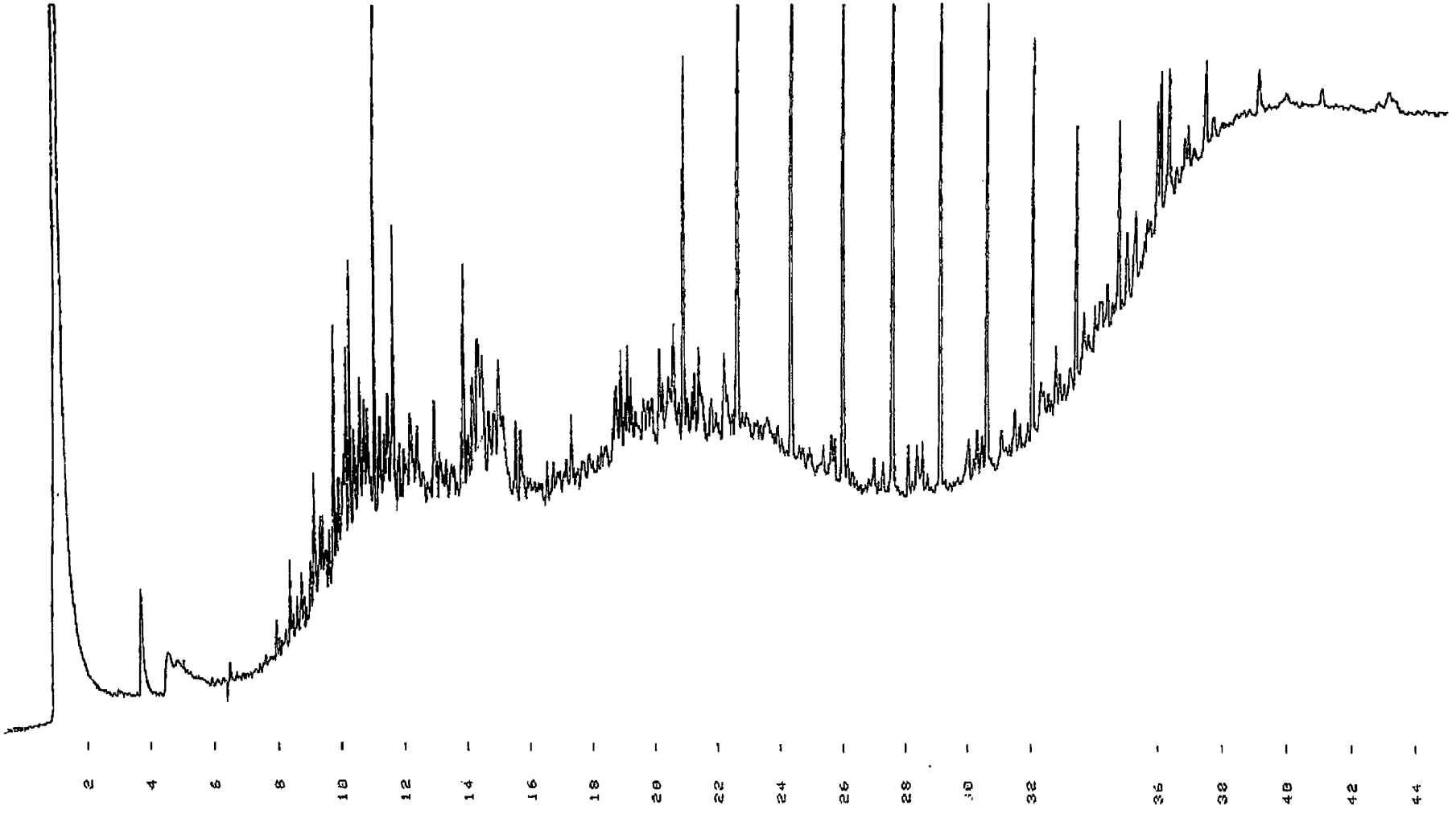

Fig. 5: Inoculated, 2wks @ 37C: peaks near 4-min are smaller than control and peaks in the time range 14-16 min are larger than control.

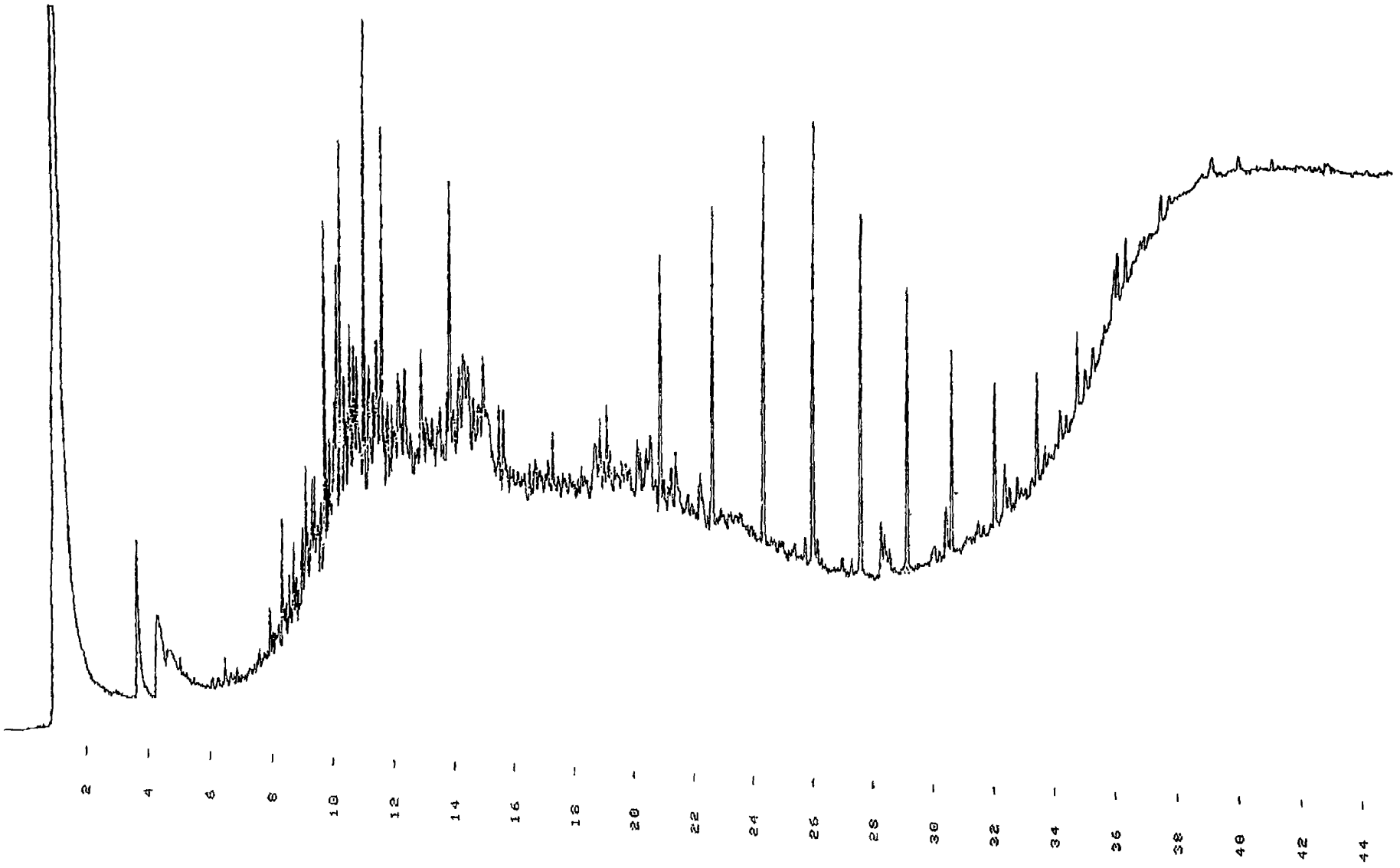

Fig. 6: Control, 2wks @ 37C: peaks near 4-min mark are larger than inoculated and peaks in time range 14-16 min are smaller than test.

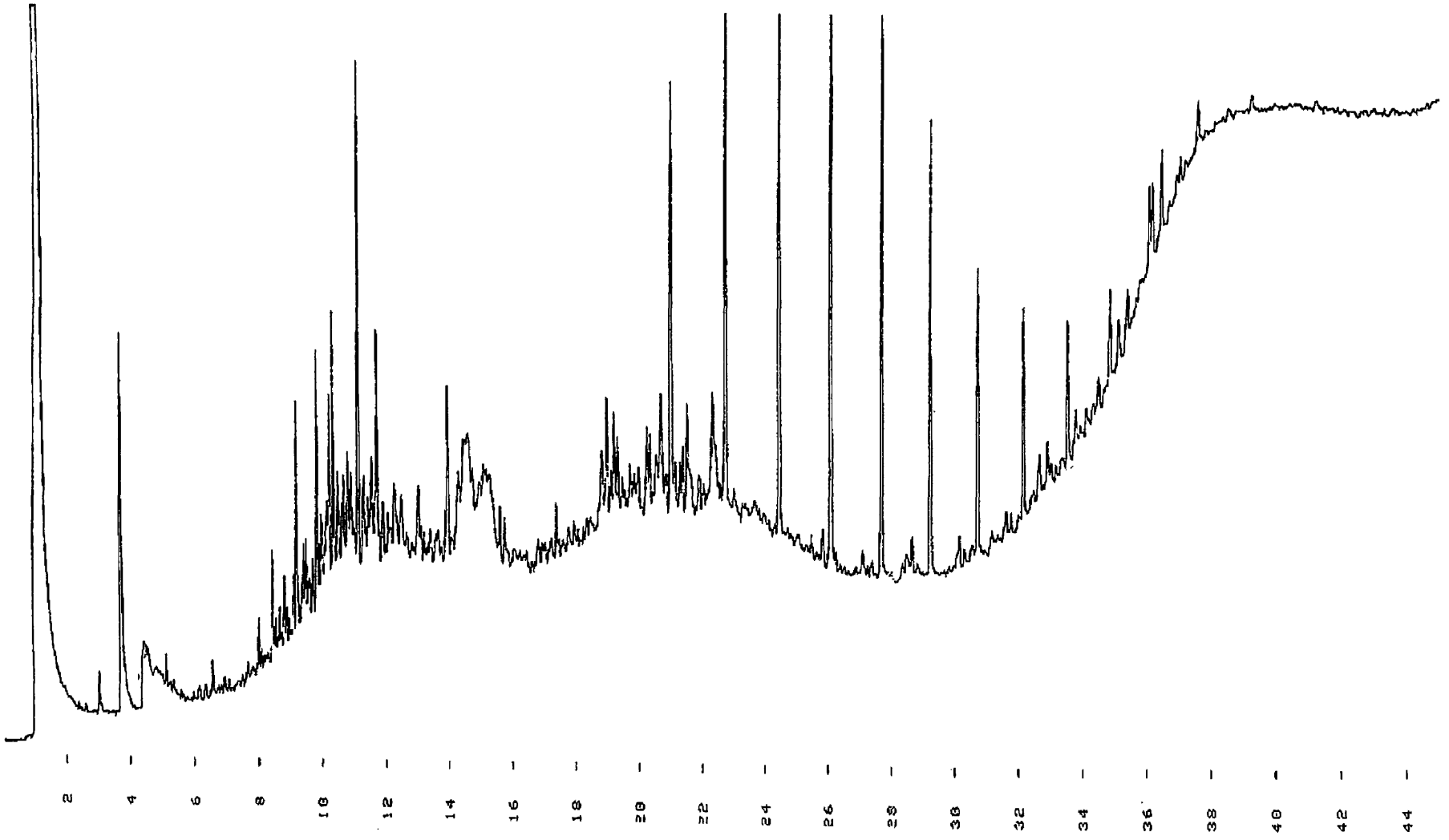

Fig. 7: Inoculated, 2wks @ room temp: note presence of peak at 3 min and large peak at 4 min and peaks size and presence in time range 10-16 min compared to control.

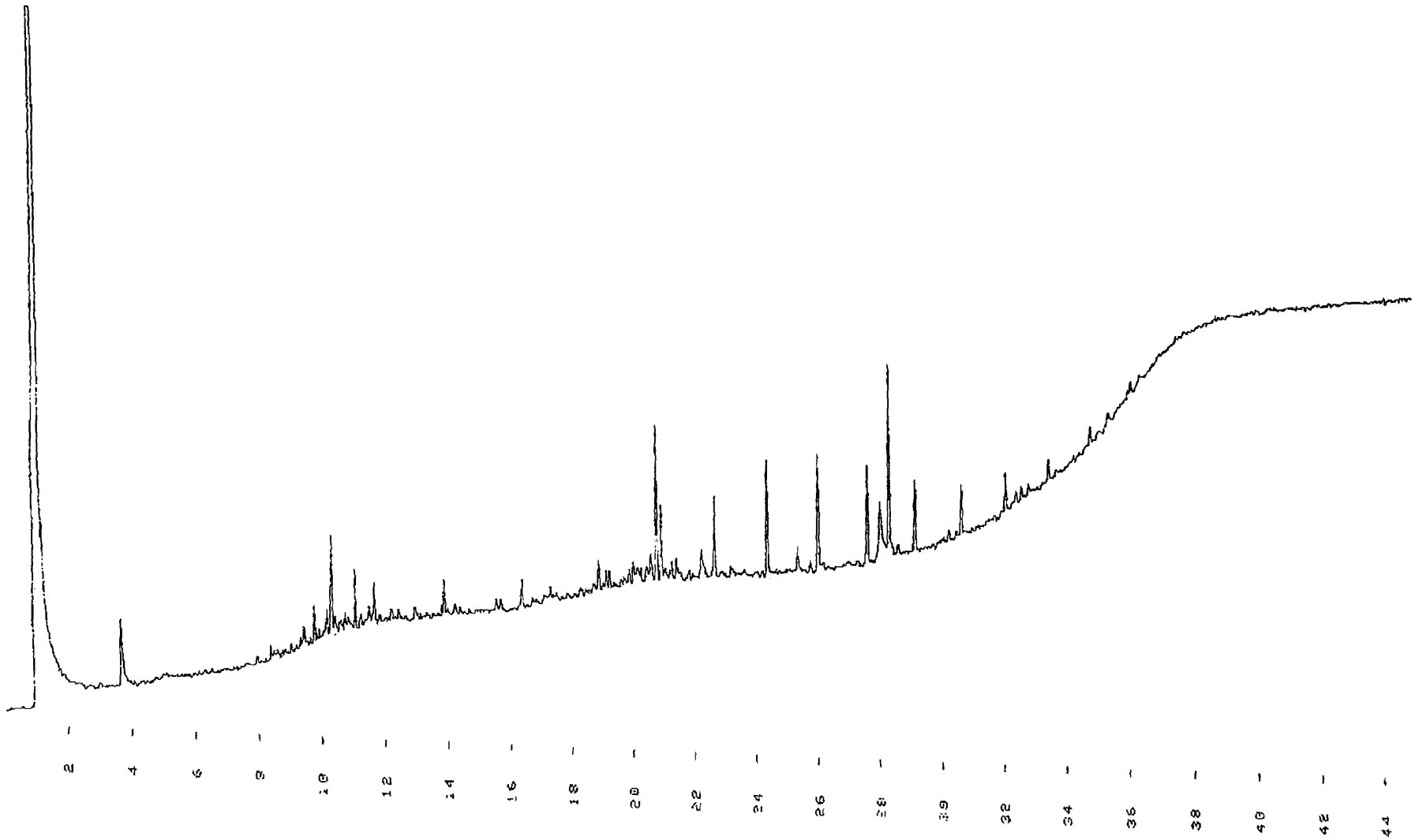

Fig. 8: Control, 2wks @ room temp: note absence of peak at 3 min and small peak at 4 min and peaks size in time range 10-16 min.

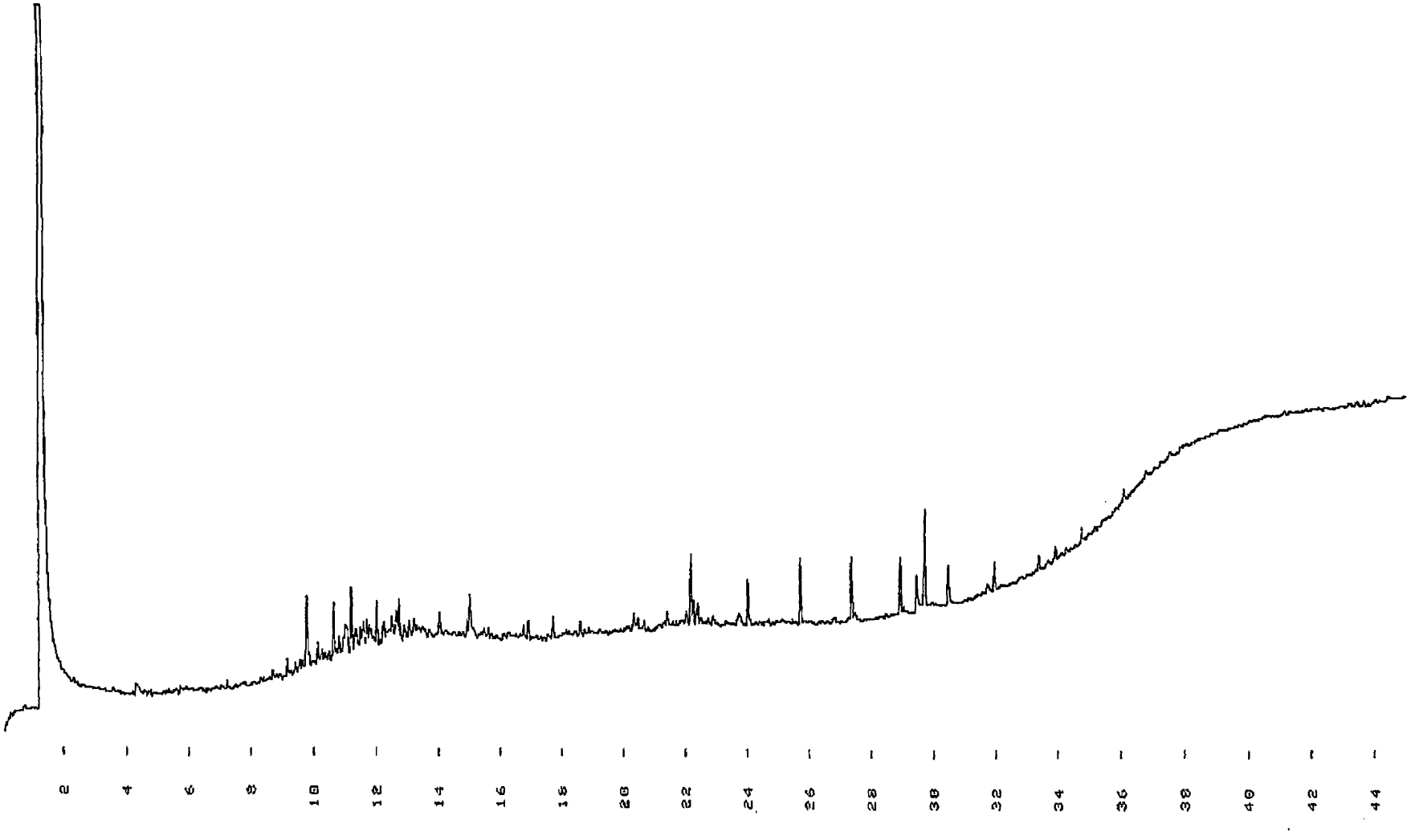

Fig. 9: Inoculated, 3wks @ 37C: note absence of peak near 4-min mark compared to control and note smaller peak size in time ranges 8-14 & 18-20.

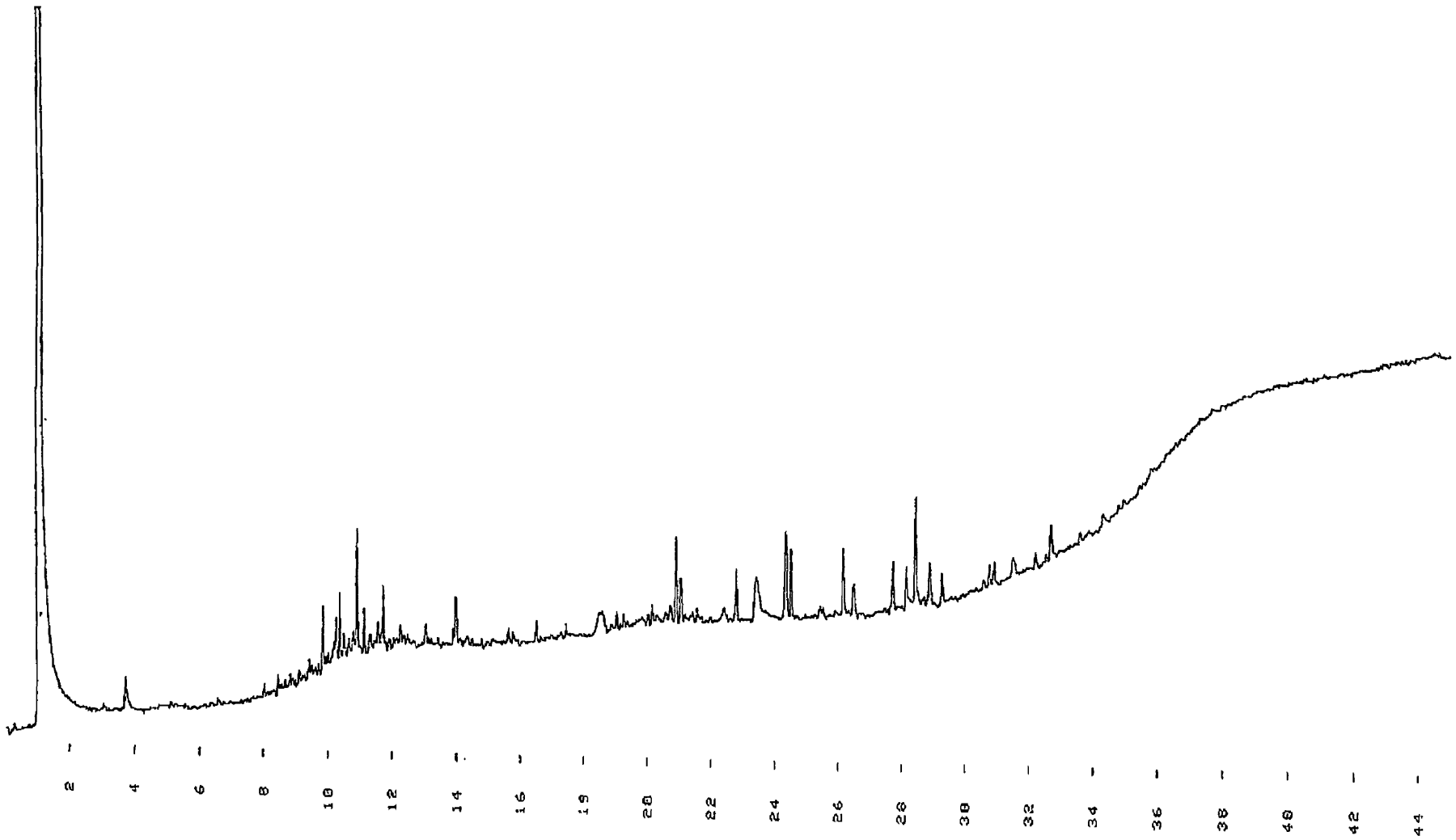

Fig. 10: Control, 3wks @ 37C: note small peak near 4 min and peak size and presence in time ranges of 8-14 & 18-20 min.

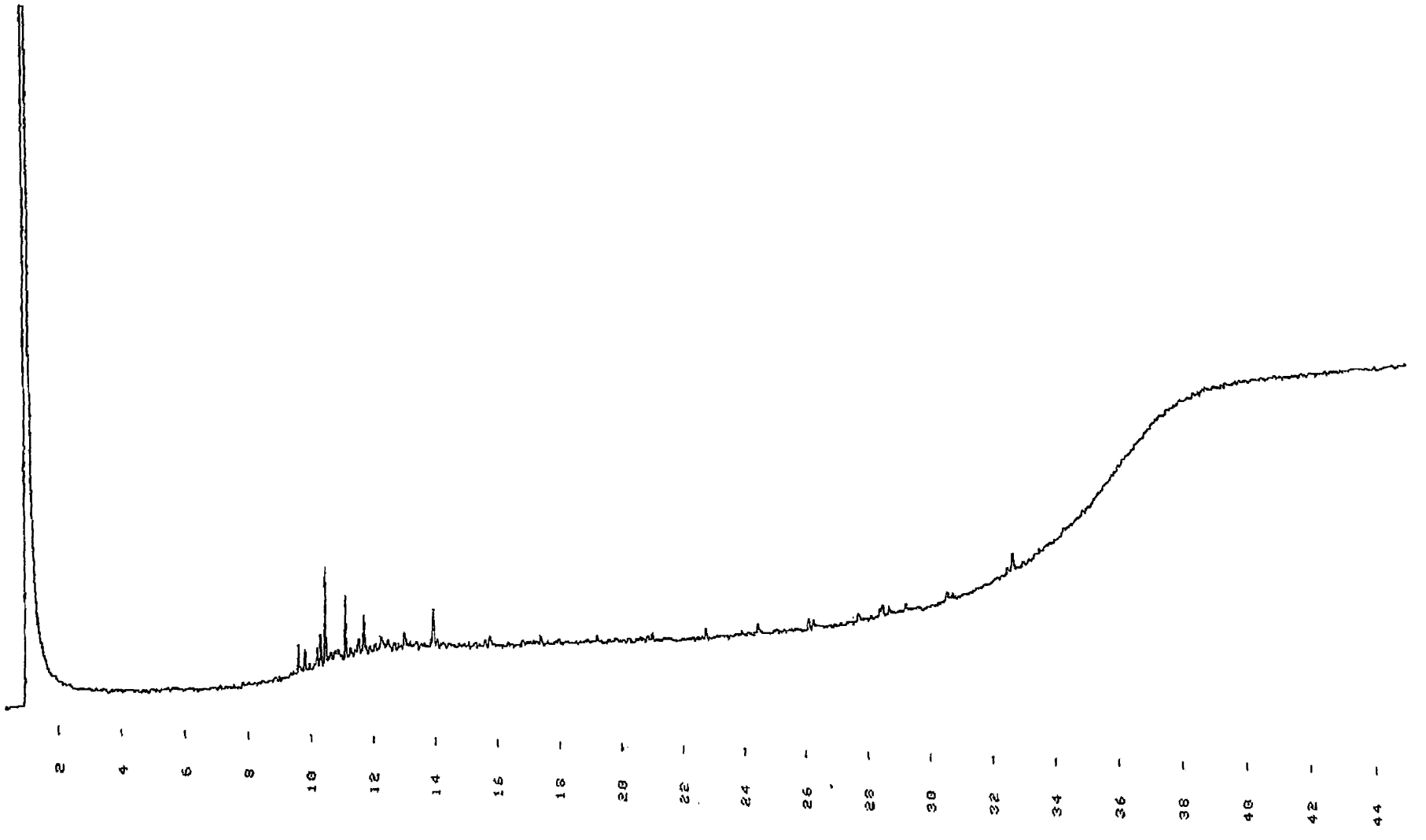

Fig. 11: Inoculated, 3wks @ room temp: note the absence of peaks at 20, 21, & 28 mins.

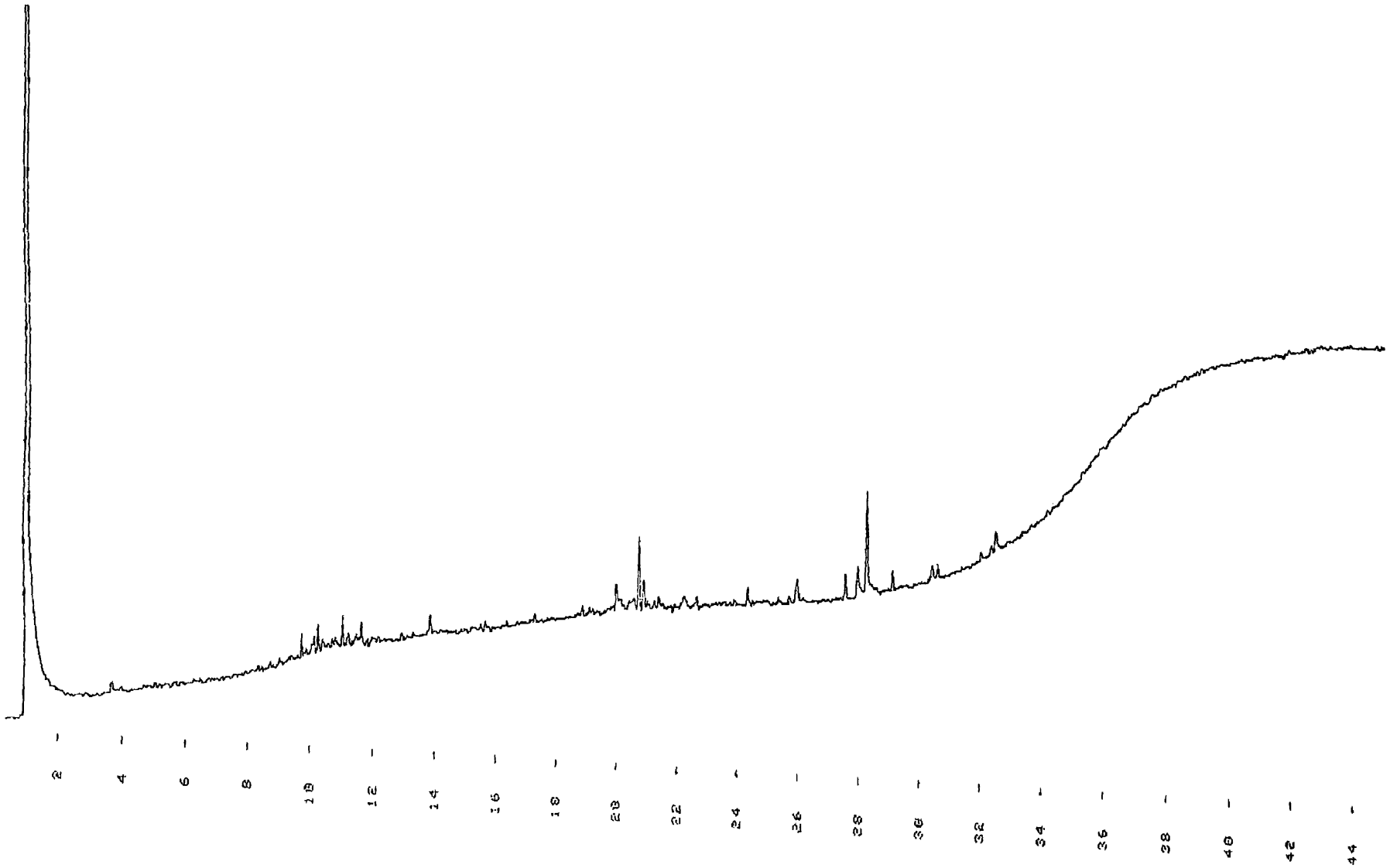

Fig. 12: Control, 3wks @ room temp: note peaks at minutes 20, 21, & 28 are present.

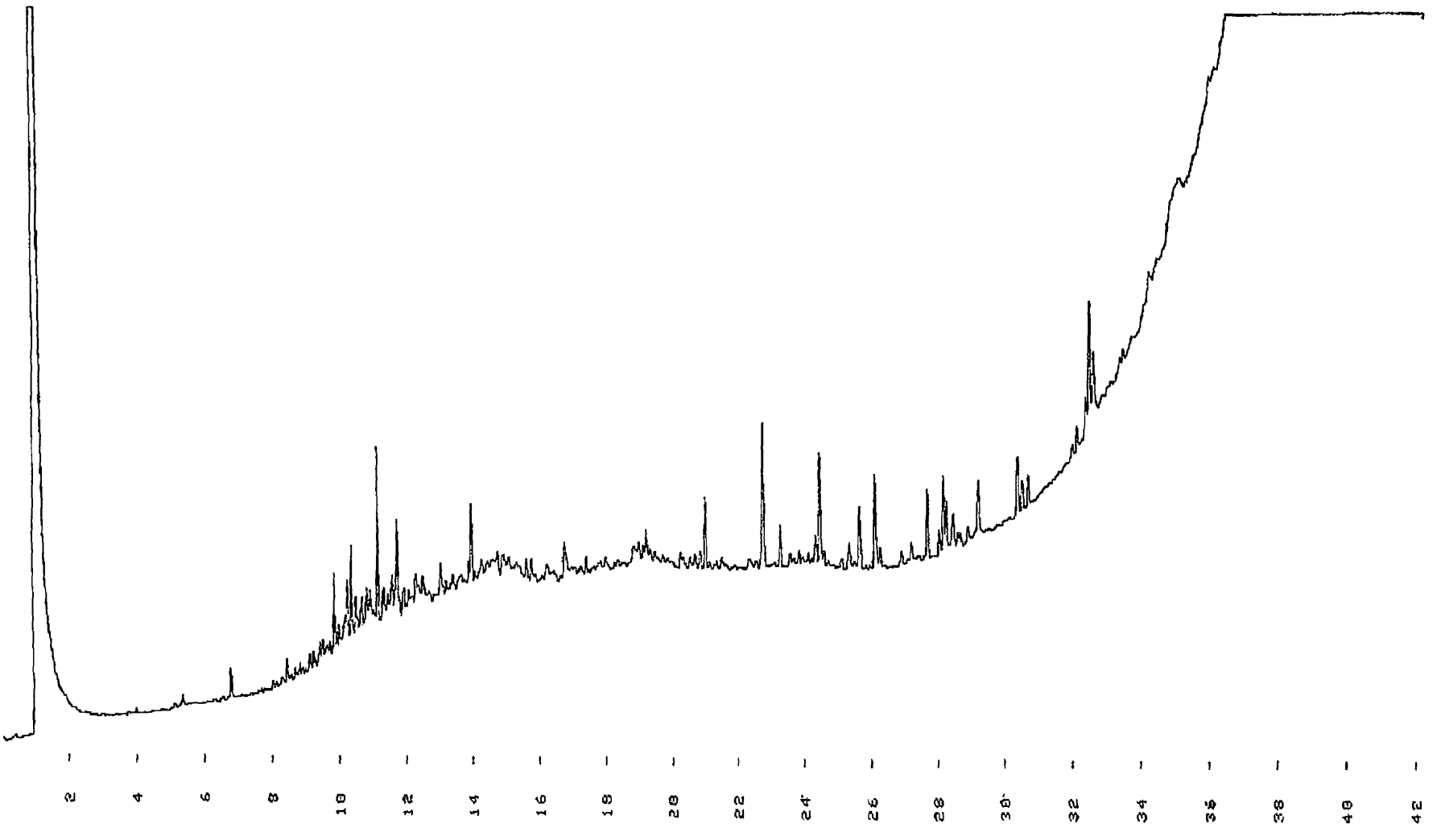

Fig. 13: Inoculated, 4wks @ 37C: note smaller peak at 11 min and smaller peaks in the time range 16-20 mon.

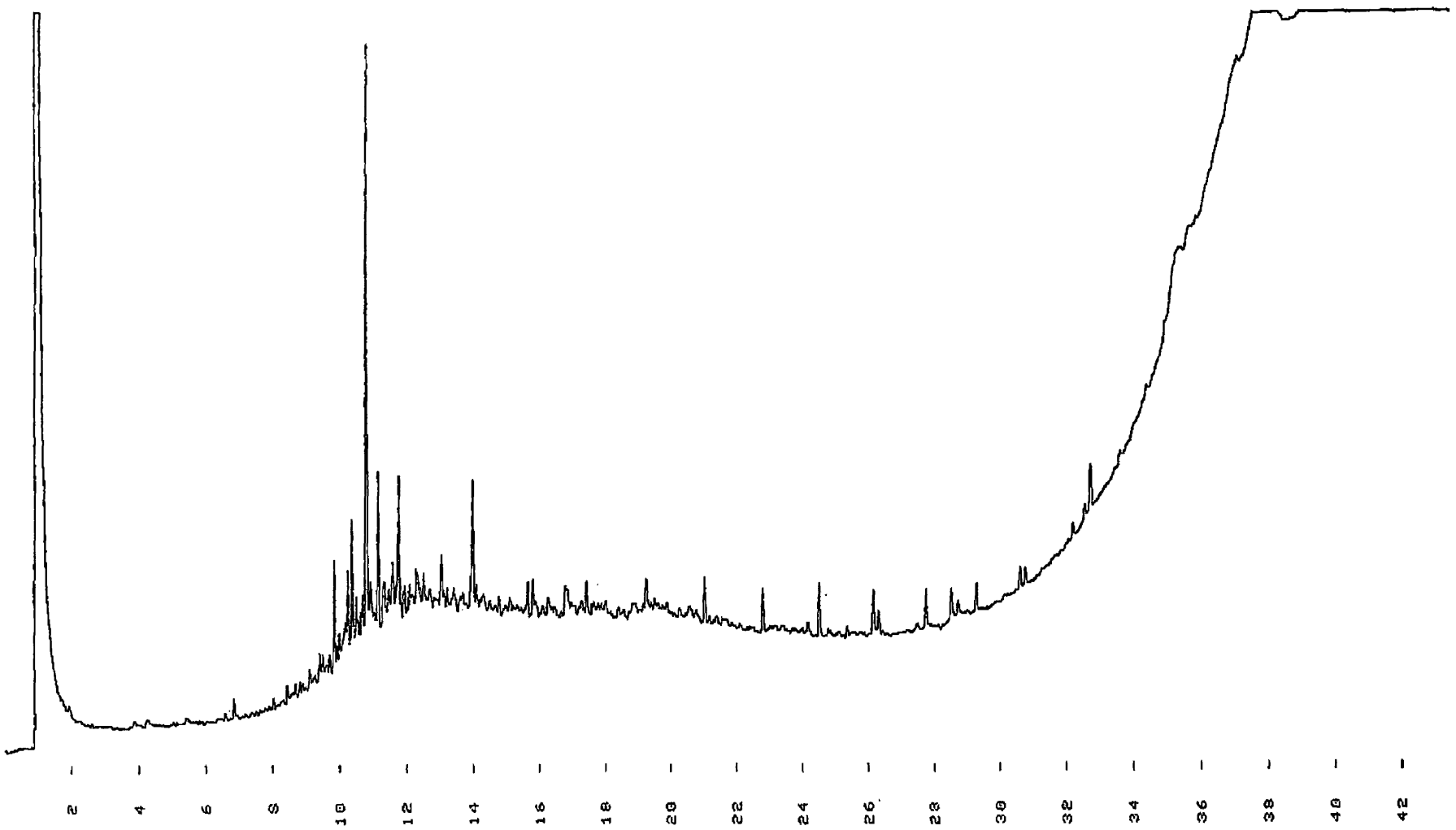

Fig. 14: Control, 4wks @ 37C: note large peak at 11 min and smaller peak at 23 min.

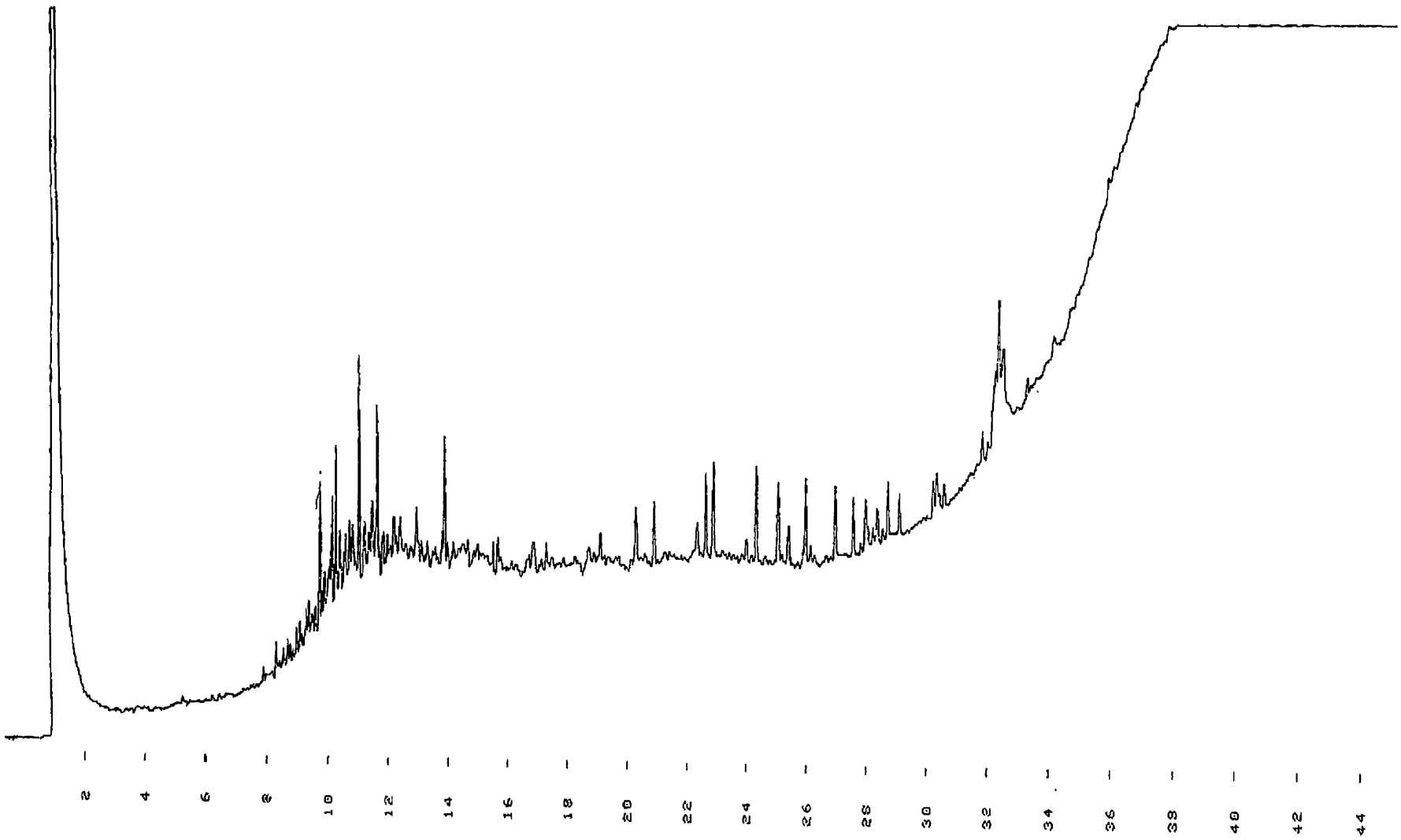

Fig. 15: Inoculated, 4wks @ room temp: note no peaks in 4-6 min and peak 20 is present and peaks are smaller.

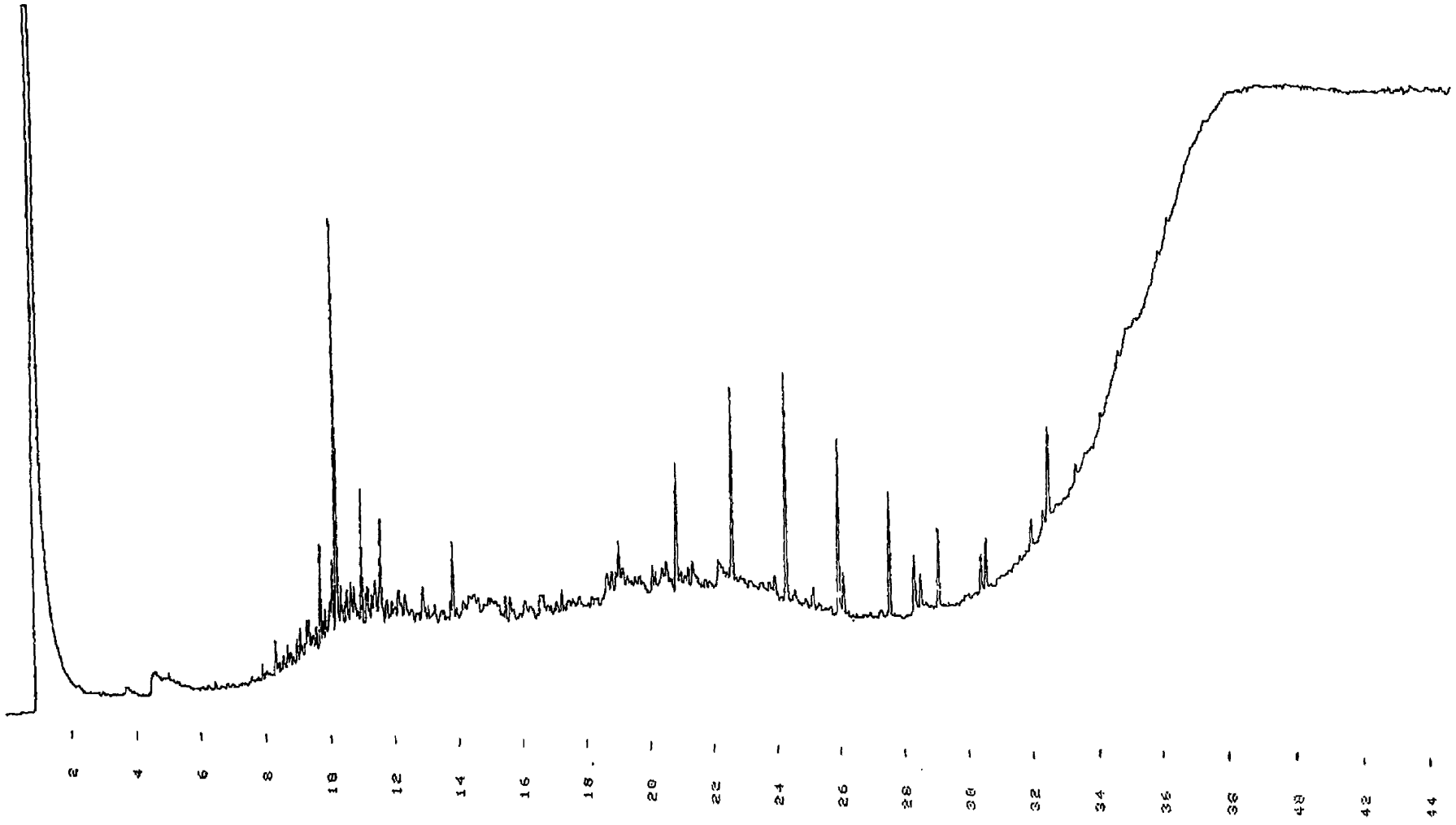

Fig. 16: Control, 4wks @ room temp: peaks appear in time range 4-6 min and peaks in control are larger than test sample.

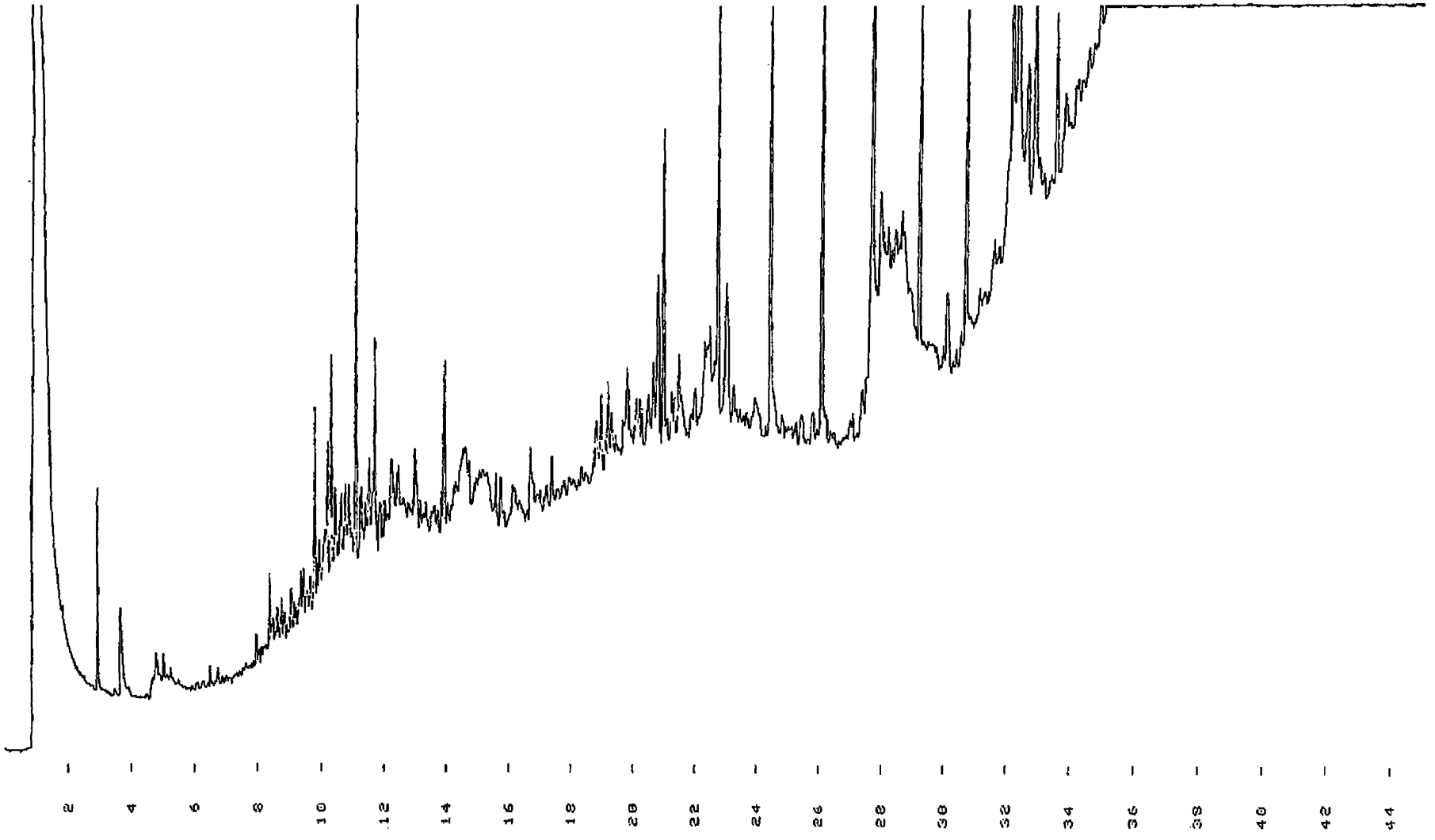

Fig. 17: Inoculated, 5wks @ 37C: note presence of peaks in 2-4 min range and larger peaks in time range 14-16 min compared to control.

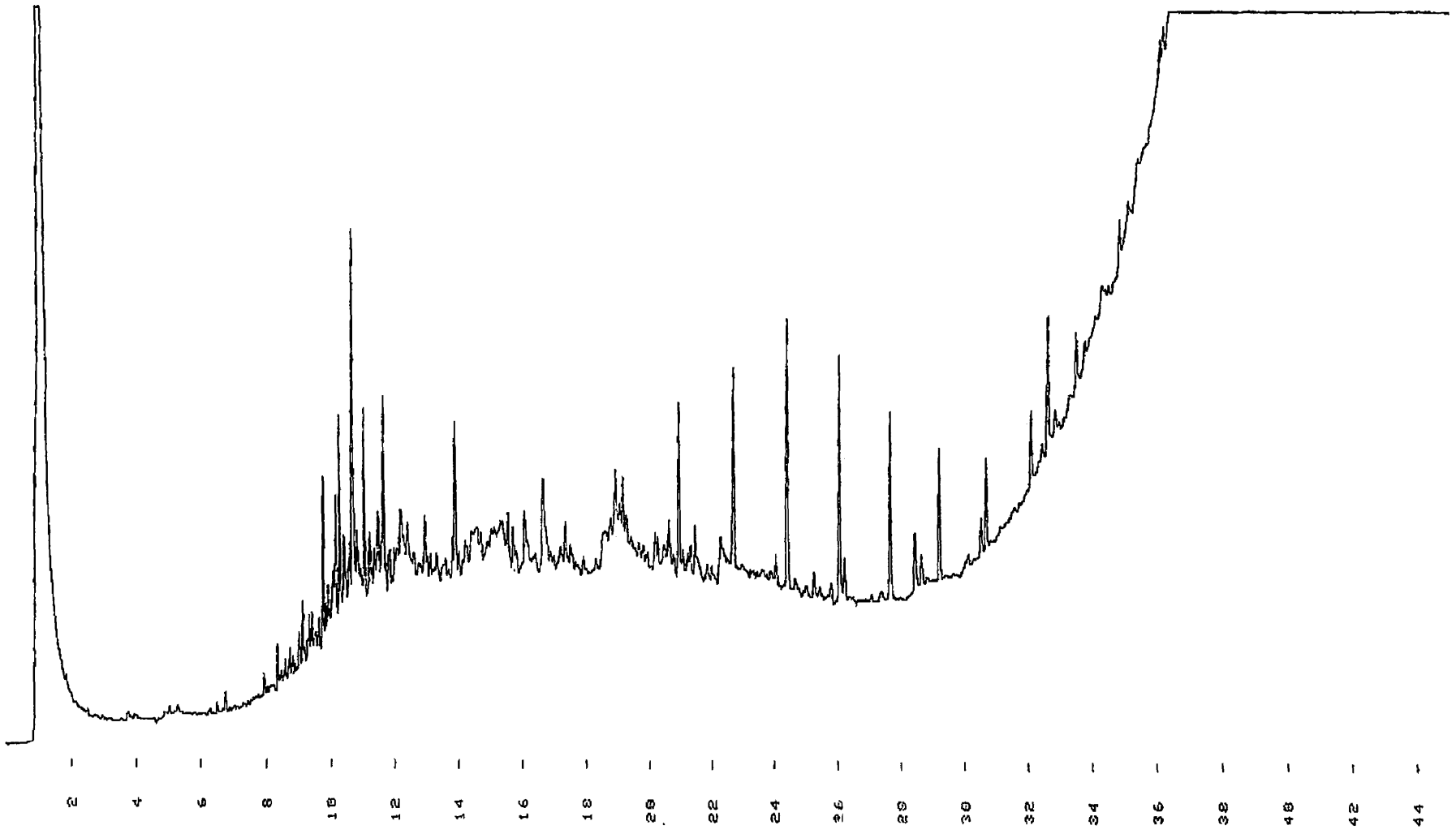

Fig. 18: Control, 5wks @ 37C: note absence of peaks in time range 2-4 min and smaller peaks in time range 14-16 min.

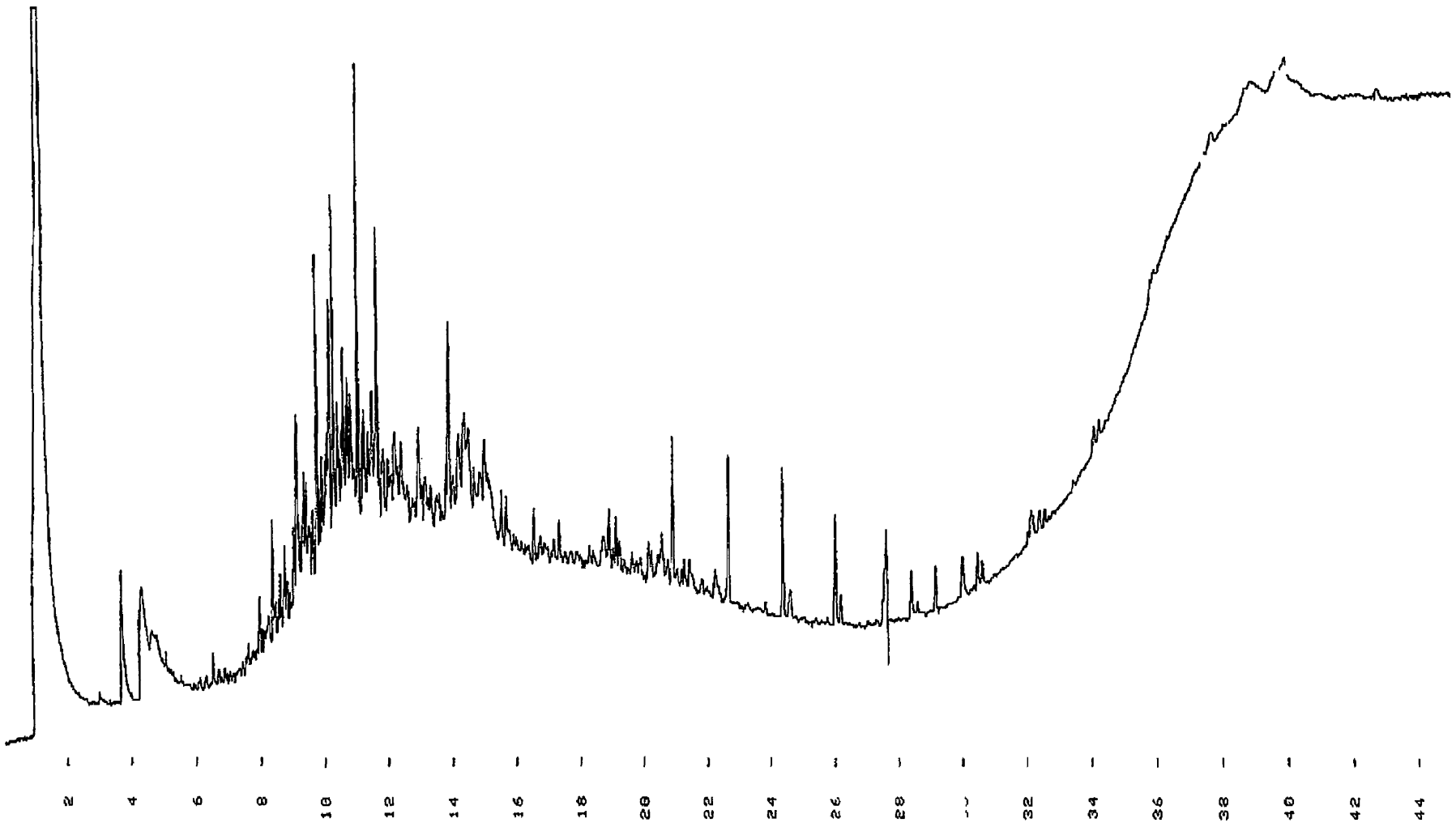

Fig. 19: Inoculated, 5wks @ room temp: note peak size in time ranges 2-6 & 14-16 min and peaks 3 & 4 compared to control.

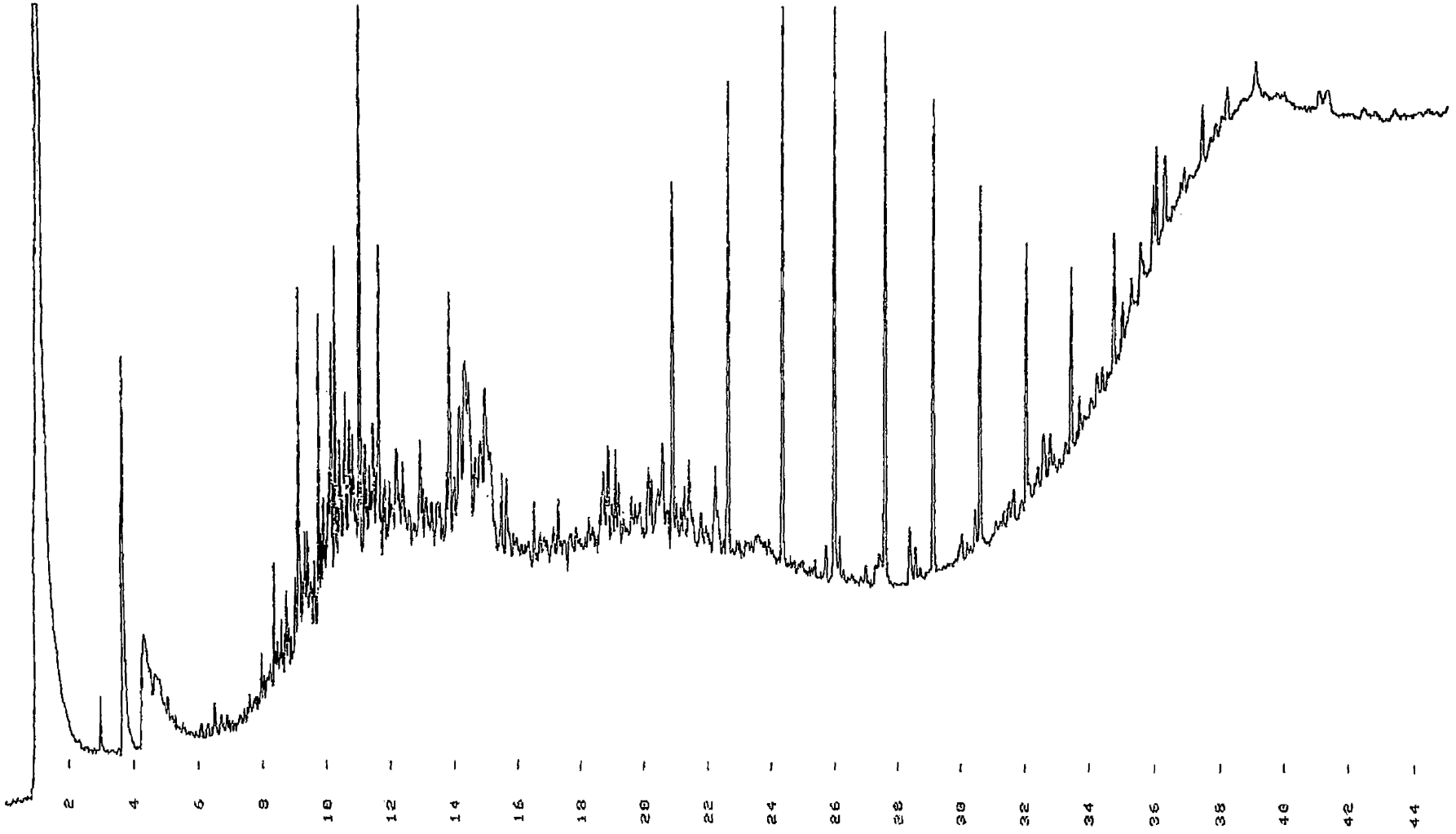

Fig. 20: Control, 5wks @ room temp: note peaks at 3 & 4 min marks compared to test.

DISCUSSION/CONCLUSION

Within the current investigation, a fungal genus was identified that could grow within a naphthenic metalworking fluid and the fungal-induced alteration of the fluid was characterized. Composition of naphthenic components of MWF is broad and loosely defined. The naphthenic condensates and oils have been shown to consist mostly of cyclohexanes and bicyclic hydrocarbons, with a small percentage of aromatics being present in the oil [15].

The composition of naphthenic MWFs can be altered by the fungus *Penicillium*. The ubiquity of *Penicillium* indicates that it can adapt to many environments. The use of MWFs in fabricating plants to cool and lubricate sheets of metal has produced thousands of liters of hazardous waste. The used naphthenic MWF is considered hazardous. The current study suggests a possible environmentally friendly way of reducing used naphthenic MWFs. The use of microbes to degrade hydrocarbons is logical because they are natural decomposers.

Markovetz *et al.* [11] showed that *Penicillium* could use alkanes and alkenes to produce moderate to good growth. They also showed that the 53 fungi tested assimilated 14-carbon chain lengths more consistently [11]. Research by Cundell *et al.* [5] looked at the mycelial structure of an unidentified species of *Penicillium* because it was capable of growing on *n*-hexadecane (and others), producing high mycelial yields. This fungus was grown in a shake-culture similar to the current investigation. However, the current investigation's liquid culture did not contain any added mineral salts. Since *n*-hexadecane has been shown to support *Penicillium* growth with high mycelial yields

and hexadecane is likely to be part of naphthenic MWFs, the observation of fungal mats at three weeks old is supported by this past research.

Research in the late 1960s showed that the species of *Penicillium* were able to alter the structure of the complex hydrocarbon naphthyridines. Naphthyridines are double ring structures similar to naphthalene except with two carbon atoms replaced with nitrogen. Hamilton *et al.* [8] showed that species of *Penicillium* could alter the chemical structure of nalidixic acid (a naphthyridine). They showed that the fungal metabolism changed the side chains of the ring structure, leaving the main ring structure unchanged [8]. Since naphthenic MWFs are composed of cyclic hydrocarbons that may have side chains and some side chains can be altered by *Penicillium*, the compounds appearing in the time range 2-4 minutes of the five week-old, 37 °C sample could be byproducts from the metabolism of the MWF.

Gas Chromatography

Khimani and Eachus' work showed that *Penicillium* is not a common contaminant of metalworking fluid [10]. Yet, *Penicillium* has been shown to oxidize two hydrocarbons, *n*-tetradecane and 1-tetradecene into alcohols and ketones [1]. These hydrocarbons could be possible natural degradation products of naphthenic MWF in higher boiling point fractions, since at this time the exact composition of the MWF is a trade secret. Allen & Markovetz's *Penicillium* contaminant degraded *n*-tetradecane into tetradecan-2-ol, dodecan-1-ol, tetradecan-2-one, and tetradecan-4-one. The 1-tetradecene was degraded into tetradecen-4-ol, 13-tetradecen-4-ol, tetradecen-3-ol, 13-tetradecen-4-one, and tetradecen-3-one. Based on their data, these alcohols and ketones were observed in the temperature range of 60-150 °C (1-10 minutes) and near the 220 °C (24 minute)

mark. In the current investigation, peaks were observed in the same temperature range (Fig. 13 and Fig. 17), and therefore could be similar alcohols and ketones, thus verifying the degradation process.

The *Penicillium* at room temperature is altering the composition of the MWF at two weeks old, producing unique peaks at retention times of 3, 4, 14, and 18-22 minutes. *Penicillium* also changes the composition of MWF at factory operating temperature (37 °C). The presence of *Penicillium* at 37 °C and at the age of two weeks old, may play a role in reducing the natural breakdown of the MWF compounds near 4 minutes (90 °C) or the fungus may be using the earlier natural breakdown products as an energy source (Figs. 5 and 6).

After two weeks at room temperature, *Penicillium* produces a larger amount of the compound at four minutes (Fig. 7). The compound that appears as peak 3 (boiling point of 80 °C) is observed at room temperature and two-weeks old with the contaminant. It is an early degradation product caused by *Penicillium*, because it is not observed in a control GC until the MWF is five weeks old (Fig. 7 compared to Figs. 17-20). Also, based on the GC, the presence of *Penicillium* does change the composition of naphthenic MWF. Figure 7 shows many peaks whereas Figure 8 (the control) shows a limited number of peaks.

Samples at three weeks old and at 37 °C showed peaks 4 (bp 90 °C) and 11 (bp 155 °C) are in the control; this observation suggests that they are compounds that are produced from the natural degradation at three-weeks-old and operating temperature. At this age, the fungal contaminant has changed the MWF composition by producing compounds that appear at the boiling point temperatures of 175 °C (peak 15) and 210 °C

(peak 22) on the gas chromatograph (Fig. 9). This time frame shows a decrease in the chemical composition of the MWF by a decrease in peak size and occurrence. The contaminant at room temperature incubation and three weeks old has decreased peaks at 20 (bp 200 °C), 21 (bp 205 °C), and 28 (bp 240 °C) minutes. This data may also suggest that the increase in temperature promotes degradation of the naphthenic MWF by *Penicillium* as compared to room temperature.

Four-week-old samples had few noticeable changes compared to the control. However, the most noticeable change to the MWF composition was the decrease of peak 11 (bp 155 °C) in the presence of the contaminant *Penicillium* (Figs. 13-16). The GC showed peak size and, at 37 °C, the relative percentage of the total area for time range 4-12 minutes (bp 90-160 °C) decreased and time zone 28-36 minutes (bp 240-280 °C) also decreased (Table 5). Decreases in the areas were also observed for the room temperature sample at age four weeks (Table 6).

Penicillium in naphthenic MWF at the age of five weeks old and at factory operating temperature showed a decrease in the areas of compounds in the boiling point ranges above 90 °C (Table 7 and Figs. 17 and 18). The control's boiling point fraction of 240-280 °C (28-36 minutes) had a percentage of 23.99, whereas the test's percentage was 15.3 (decreased by 36%). An approximate decrease of 26% also occurred in the 90-160 °C boiling point fraction (test 7.68 and control 10.44). However, for the earlier time range of 2-4 minutes (bp 70-90 °C), the area percentage increased. This increase in the percentage of the peaks could be a result of compounds (with boiling points above 90 °C) being decomposed into smaller compounds, which have boiling points in the earlier time range. Overall, based on the gas chromatographs at these two temperatures and ages, the

increase in temperature from room (~23 °C) to operating temperature (37 °C) increases the fungal activity in naphthenic MWF.

Relevance of Penicillium *to Health*

Penicillium is a universal component of the soil and is distributed worldwide by wind-blown spores. By being so easily distributed in soil, it can change locations from soil to air to household dust rapidly [26]. Species of *Penicillium* are mostly known for being found in damp or water damaged buildings and may be found in stored foods, grains and on fruits. Some species of *Penicillium* are used in food production, such as the production of cheese. The abundance of this genus of fungi coupled with some of the genus members having the ability to produce food-contaminating toxins make it a concern for human and animal health. These mycotoxins (toxins produced by fungi) may have varied effects on people such as simple food poisoning or, in some cases, far worse, such as neurological disorders or death.

However, the contaminated MWF emulsion was associated with a red irritated skin rash present on some workers who had been exposed to the fluid (G. O'Dell, personal communication). Definitive evidence that the rash is a result of exposure to the fungus or agents within the MWF is still lacking.

As presented earlier, MWFs provide a habitat for microorganisms. These industrial fluids allow for a variety of organisms to live including pathogenic species. Rossmoore *et al.* [14] identified the species that were growing in an automotive engine plant that experienced acute outbreak of myalgia and fever. Their work identified over 30 species of bacteria, nine species of yeast and four genera of filamentous fungi (one of which was *Penicillium*). They showed that there is a great potential for MWFs to shelter

microorganisms, which could cause sickness in many people. Their research also showed that mold and yeast counts were related to the activity of aerobic bacteria, *i.e.*, counts were high for both organisms at the same time. The above article did not identify the chemical composition of the MWF or the species of fungi in the counts.

Another idea why workers became ill is the *Penicillium* metabolites from the MWF degradation or mycotoxins. Gordon's [7] research showed that endotoxin from bacteria was associated with illness. The current study identified a fungal contaminant that was associated with cases of workers developing a rash.

In conclusion, the current study identified the contaminant as *Penicillium*. The study showed that *Penicillium* could grow in a naphthenic MWF used at a metal fabricating facility. Also, the study showed that this contaminant is capable of altering the chemical composition of the MWF as shown by the gas chromatographs. The GCs also showed that *Penicillium* could produce unique peaks (thus, compounds) in the boiling point fraction 60-110 °C (2-6 minutes). The data also showed that *Penicillium* was decreasing the areas of the different boiling point fractions. Based on the current study's data, it takes some time to decrease the relative area of the emulsion oil at different boiling point fractions with *Penicillium* alone. Although, the exact chemical changes are not known, the study has provided a starting point for future investigations of the chemical composition. Additionally, the alteration of the MWF by *Penicillium* may condition the fluid for secondary contamination by other microbial agents. Conversely, while *Penicillium* alone takes time to decrease the amount of complex hydrocarbons present in naphthenic oils degradation, concurrent microbial contamination may accelerate the process.

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APPENDIX 1

Table A: Composition of Quaker Draw #59 (provided by Quaker Chemical)

Quaker oil Draw # 59:	
Naphthenic Oil	~65 %
Petroleum type sulfonates, Ca & Na	5-10 %
Other waxes/sulfonates	8-15 %
Amine Borate reaction product (AMP)	3-10 %
Glycol Ether	1-5 %
P containing compounds	1-5 %
Emulsifiers (various) ethoxylated	1-5 %
Fatty Acid	1-5 %

Table B: Boiling Points of Selected Hydrocarbons

Chemical Name & Formula	*°C*	*Minute*
Cyclobutane, ethyl ($C_2H_5C_4H_7$)	70.7	2
Benzene (C_6H_6)	80.1	3
Cyclohexane (C_6H_{12})	80.7	3
Heptane (C_7H_{16})	98.4	4.5
Cycloheptane (suberane) (C_7H_{14})	118.5	6.5
Octane ($CH_3(CH_2)_6CH_3$)	125.7	7.5
Cyclopentanone (C_5H_8O)	130.6	8
Cycloheptane, methyl ($C_7H_{13}CH_3$)	134	8.5
Decane ($C_{10}H_{22}$)	174.1	14.5
2-decanol ($CH_3(CH_2)_7CHOHCH_3$)	211	22
Dodecane ($C_{12}H_{26}$)	216.3	23
Cyclobutane, benzoyl ($C_6H_5COC_4H_7$)	260	32
Hexadecane (centane) ($CH_3(CH_2)_{14}CH_3$)	287	37.5
***dodecylcyclohexane ($C_{18}H_{36}$)	331	46

Table B: The boiling points of selected hydrocarbons that could be found in naphthenic metalworking fluid. Boiling point values are from *CRC Press Handbook of Chemistry and Physics*, 74th edition and *** from 87th edition. Times are approximations.

Appendix 2

Identification of *Penicillium* to species level used two approaches, a software system and growth conditions on selected media plates. The software system used was BioLog (MicroLog System, Release 4.2, Haywood, CA), which evaluates the biochemical activity of the sample. The possible species from BioLog were *P. brevicompactum*, *P. vulpinum*, or *Eupenicillium euglaucum*. These species had a higher percent of similarity.

The second approach used the growth conditions established by Pitt (Pitt, J.I. 1979. *The Genus* Penicillium *and Its Teleomorphic States* Eupenicillium *and* Talaromyces. Academic Press, Ltd., London, United Kingdom.) This technique used three different media at three different temperatures. Two important growth characteristics of the fungal contaminant are (1) no growth / no germination on Czapek Yeast Autolysate agar (CYA) at 5 °C and (2) colony diameter on CYA at 37 °C exceeded 20 mm. Based on the above characteristics and others, the possible species from Pitt were *E. abidjanum*, *E. javanicum*, *E. brefeldianum*, *E. hirayamae*, *E. ehrlichii*, *E. terrenum*, *P. donkii*, *P. chermesinum*, *P. oxalicum*, or *P. piceum*. The current study contaminant *Penicillium* had some characteristics similar to each of the above species.