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A calorimetric analysis of soil treated with effective microorganisms

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ABSTRACT

Effective microorganisms or EM is a consortium of beneficial microorganisms (primarily photosynthetic and lactic acid bacteria, yeast, actinomycetes, and fermenting fungi) that can be applied as inoculants to increase the microbial diversity of soil. Two fields of sugar cane in the province of Tucumán, Argentina were treated with either EM containing brewery fermentation tank residual yeast (YS) or just EM (NYS). The outcome was 1000 kg Ha^{-1} less of sugar cane harvested from YS field. The aim of this work was to analyze both soils to find out if calorimetry could assess the negative effect of yeast. Mass specific thermal power–time curves were obtained during 100 or more h of measurement. Once a day, a vial containing NaOH solution was introduced into the calorimetric ampoule to determine CO_2 evolution. A plot of log SMB (soil microbial biomass) as a function of log p gave a similar relation as previously reported for those samples that showed an energy expenditure of 16–22 J cm^{-3} CO_2 respired. Thus, soil microbial biomass could be determined during microbial growth. Results showed that yeast suppressed the activity of part of the soil microorganisms. Also, soil microbial activity increased with time in NYS due to successive applications of EM.

1. Introduction

Agriculture is sustained nowadays by means of intensive use of agrochemicals in order to preserve soil quality and to obtain high yields. Meanwhile, the international market increasingly requires natural or organic food, free of chemical residues. As a result, alternative agricultural systems such as the use of beneficial microorganisms are being developed with the aim to improve and maintain soil quality. Among such microorganisms is the fungi genus *Trichoderma*. Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants [1]. The use of Effective microorganisms (EM) is done on a smaller scale.

Dr Teruo Higa, an horticulture professor at the University of Ryukyus, Okinawa, Japan discovered and developed the EM technology [2]. EM is a mixed consortium of beneficial microorganisms of natural occurrence (lactic bacteria, yeast and photosynthetic bacteria, among others) that can be applied as an inoculant to increase the microbial biodiversity of soils and plants [3]. EM is not a substitute for other cultural practices; it is an additional tool to optimize the best practices of soil and crop handling such as crop rotation, utilization of organic amendments, conservation tillage, recycling of harvest residues and pest biocontrol. If used in an appropriate manner, EM can significantly increase the effects of these practices [4]. Originally, EM was developed as an alternative to pesticides and chemical fertilizers. However, in the last two decades the utilization of EM has expanded to the treatment of water and wastewaters, odor control, farm and animal health, human health many industrial treatments [5]. This consortium of microorganisms produces accelerated breakdown of proteins, sugars, fats and fibers, and thus, promotes rapid organic matter decomposition. EM works in two primary ways: (i) competitive exclusion of harmful microorganisms and (ii) by production of beneficial products such as enzymes, organic acids, amino acids, hormones and antioxidants that promote the health of the environment [6].

EM technology is used in a farm located in the southwest of Tucumán province, Argentina. However, during 2011 besides EM, the farmer tried EM composted with the residual yeast (*Saccharomyces* spp.) obtained from brewery fermenting tanks in a sugar cane field. The outcome was 1000 kg Ha^{-1} less of sugar cane than in the field treated with just EM. The aim of this work was to find out, by means of isothermal calorimetry: (i) how the yeast affected soil microbial activity and (ii) the effect of EM on soil microbial activity.

2. Materials and methods

2.1. Experimental site

A field experiment was done on a farm located southwest in the province of Tucuman, Argentina (27°45′ S, 65°39′ W) in 2009.

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The soil was sandy loam with the soil characteristic (0–30 cm) of 57.7% sand, 30.8% silt and 11.6% clay. The field was planted with sugar cane. Annual mean air temperature was 22 °C and average precipitations was 740 mm (2010/2011) and 690 mm (2011/2012).

2.2. Experimental treatments

The commercial preparation of effective microorganisms (EM) was activated by fermentation in vinasse (a sugar cane industry residue) to obtain EMA when pH was below 3.5. Then, this EMA was dispersed on the soil with water (140 $dm^3\ Ha^{-1}$). The yeast residue was obtained from 'Quilmes' Brewery, Famaillá, Tucumán, Argentina and composted 4–5 days with EMA prior to being sprayed on the soil. The experimental site was divided in two lots and treated as follows: (A) lot YS: 8/2010: 3.5 $dm^3\ Ha^{-1}$ EMA, 10/2010: 7200 $dm^3\ Ha^{-1}$ residual brewery yeast and then EMA as follows: 12/2010: 4.5 $dm^3\ Ha^{-1}$, 10/2011: 10 $dm^3\ Ha^{-1}$, 11/2012: 20 $dm^3\ Ha^{-1}$, 8/2012: 10 $dm^3\ Ha^{-1}$. (B) Lot NYS only EMA as follows: 11/2010: 4 $dm^3\ Ha^{-1}$, 12/2010: 4.5 $dm^3\ Ha^{-1}$, 9/2011: 20 $dm^3\ Ha^{-1}$, 11/2011: 10 $dm^3\ Ha^{-1}$, 8/2012: 10 $dm^3\ Ha^{-1}$.

2.3. Soil material

Ten sites were randomly chosen and sub-samples were collected up to a depth of 15 cm (A horizon = 20 cm), after removing the top layer. After combining the sub-samples, they were sieved (2 mm × 2 mm) to remove root residues and coarse material and stored in polyethylene bags at 5 °C until used.

2.4. Chemical and microbiological soil analysis

Water content (WC) was determined by drying an aliquot (×2) until constant weight at 105 °C [7]. Bulk density and field capacity humidity (FCH) were determined by the graduated cylinder method [8]. The pH was measured with a glass electrode on a suspension of soil in deionised water (1:1) [8]. Organic carbon (OC) was determined by wet oxidation with $K_2Cr_2O_7/H_2SO_4$ [9]. Extractable phosphorus (P) was photometrically determined by the Olsen extraction method [10]. Extractable nitrogen as $NO_3^- + NO_2^- + NH_4^+$ was determined by the diffusion method [11]. Colony forming units (CFU g^{-1}) were determined by the serial dilution method by using trypteine soy agar (TSA) as culture media.

2.5. Calorimetric analysis

A twin heat conduction calorimeter (Lund University, Sweden) was used [12,13]. Soil samples (3.0–4.0 g dw) were stabilized during 7 days at 25 °C in a polyethylene bag at a water content equivalent to 60% of FCH. Then, an appropriate amount of water containing glucose as to get FCH and the required amount of the carbon source was added. The soil was thoroughly mixed by hand and the equivalent of 1.0–1.5 g (dw) was weighed into the calorimeter ampoule (8.0 cm^3). The ampoule was hermetically closed and after the 30 min needed to equilibrate the calorimetric system, thermal power (P) – time (t) curves of microbial growth were recorded at 25 °C. An ampoule containing 1 g of agar was used as reference. Microsoft Excel 2007 (Microsoft corporation) and Origin 6.0 (Microcal, Inc.) were used to convert the curves obtained into mass specific thermal power (p) – time (t) curves and integrated to obtain the specific heat (q) associated with the glucose degradation. From the semi-logarithmic conversion of the portion of the curve that indicates exponential microbial growth ($\log p = \log p_0 + \mu t$) the microbial growth rate constant, μ, was calculated. To determine the enthalpy change due to metabolism the expression $\Delta_m H = (q/m)$ 180.16 was used where m is the amount of glucose used and 180.16 is the molecular weight of glucose. In turn, $\Delta_m H$ results from an enthalpy change due to catabolism, $\Delta_{cat} H$, and an enthalpy change due to anabolism, $\Delta_{an} H$ expressed as $\Delta_m H = \Delta_{cat} H + \Delta_{an} H$.

2.6. Calorespirometry

Calorespirometric assays were performed by using soil (1–1.5 g dw) at FCH with and without glucose. After the system was equilibrated and values of thermal power (P_1) recorded during 1–2 h, a vial containing a solution of 1.0 M NaOH (trap of CO_2) was introduced and values of P were collected again (P_2). After collecting data for 2–3 h, the vial was removed, and metabolism was measured again (P_3) [13,14]. This process was performed once or twice a day during one week. Then, the values of P were converted into specific values (p) by dividing by the dry mass of the sample. The specific rate of CO_2 evolution, rCO_2, was calculated by using the expression: $rCO_2 = \{p_2 - [(p_1 + p_3)/2]\}/109.4$. The value of −109, 4 kJ mol^{-1} is the heat of reaction of CO_2 with 1.0 M NaOH to produce CO_3^{2-} [15]. The value of rCO_2 expressed in $cm^3\ kg^{-1}\ h^{-1}$ is used to calculate soil microbial biomass, SMB [13] by using the conversion factor of 32.4. The ratio p/rCO_2 is a measure of the efficiency of carbon conversion from glucose into biomass [16,17]. The $p1$ value was used to calculate this ratio.

3. Results and discussion

Table 1 shows the physicochemical properties of both soils collected in 2011 and 2012. Note the higher pH for YS as compared with NYS in 2011 in both years. Thus, the higher pH of YS must be due to the yeast residue.

A remarkable note was the increase of OC content of both soils over just one year (20%) in situ. This must be due to the frequent applications of EMA. On the contrary, YS stored at 4 °C during 1 year lost 17% OC whereas NYS kept the original value. This might indicate a mineralization process in YS at the temperature of storage. A study of carbon and nitrogen mineralization in soil after the addition of compost – made of brewing yeast and lemon tree prunings – showed there was a high release of CO_2 at early stages of biostabilization. Mineralization of organic carbon was around 25% in 70 days of incubation [18]. In our work, the composting process was just 4 to 5 days. The same probably did not occur in the field due to EMA applied after the yeast, thus neutralizing the negative effect of yeast.

Something striking was to find no CFU g^{-1} in newly collected soil (Table 1). Only after 1 year of storage, colonies were possible to be counted. Fig. 1A and B shows the $p - t$ curves obtained during 25 h for both soils at FCH supplemented with different amounts of glucose. Specific thermal power (p) – time (t) curves obtained with soil amended with glucose did not show the typical shape of microbial growth curves.

These curves show that the optimum concentration of glucose for YS was 5 mg g^{-1} (curve c of Fig. 1A) due to the higher p values. However, the optimum glucose concentration for NYS was 3 mg g^{-1} as observed in Fig. 1B, curve a. Due to the strange shape of these curves, we decided to measure specific thermal power during 100 or more hours and also, to measure the evolution of CO_2 once or twice a day. Both soils were tested at different periods of time during 1 year. Fig. 2 shows the $p - t$ curves obtained.

Note the similarity among the $p - t$ curves of each soil during 2011 (Fig. 2, curves a–c). However, a difference can be observed between the soils. The most striking difference was that of YS and NYS collected in 2012 (curve d, Fig. A and B, respectively). The microflora responsible for the peak at 80 h in NYS is not present in YS.

Table 2 shows the thermodynamic parameters calculated from the curves in Fig. 2.

Table 1
Values of field capacity humidity (FCH), pH, organic carbon (OC), nitrogen (N as $NO_3^- + NO_2^- + NH_4^+$), extractable phosphorous (P) and colony forming units (CFU g^{-1}) for the soils studied.

Sample	FCH/%	pH	OC/mg kg^{-1}	N/mg kg^{-1}	P/mg kg^{-1}	10^{-7}CFU g^{-1}
NYS 2011	20.0	6.01	16.9 ± 0.8	10.7 ± 0.6	43.7 ± 3.9	None
NYS2011[a]	–	–	18.3 ± 0.1	–	–	5.31 ± 1.17
NYS2012	23.3[**]	5.85[**]	19.5 ± 0.3[**]	17.1 ± 2.1[**]	33.3 ± 0.5[**]	None
YS2011	20.0	6.33	17.5 ± 0.9	8.5 ± 0	27.5 ± 3.1	None
YS2011[a]	–	–	15.0 ± 0.4[**]	–	–	3.05 ± 0.65
YS2012	27.1[**]	6.05[**]	21.7 ± 1.4[**]	17.1 ± 2.1[**]	26.1 ± 0.3	None

[a] Analyzed after 1 year of storage.
[**] Significantly different at 0.05 confidence level.

Table 2
Heat evolved (q) during the degradation of glucose (m) by microorganisms that grow at a rate constant (μ) producing a peak time (t_p) with a mass specific thermal power (p_t). The whole reaction results in a metabolic enthalpy change ($\Delta_m H$) that produced soil microbial biomass change (ΔX).

YS: M/Year	m/mg g^{-1}	$-q$/J g^{-1}	t_p/h	$p_t/\mu W\,g^{-1}$	$-\Delta_m H$/kJ mol^{-1}	μ/h^{-1}	$\Delta X/\mu g\,g^{-1}$
4/2011	3	30.1	38.2	90.2	1808	–	127
7	3	26.5	59.4	96.2	1447	–	476
12	5	40.4	51.4	131.2	1446	–	274
4/2012	5	52.8	25.4	258.0	1902	0.026	719
NYS: M/Year							
4/2011	3	47.7	–	125.0	2865	–	294
7	3	37.6	7.4	171.2	2258	–	496
12	3	46.7	83.7	101.1	2805	–	268
4/2012	5[a]	–	31.8	190.4	–	0.028	837
	5[b]	74.6	79.9	278.5	2688	0.019	608

[a] Corresponds to first peak.
[b] Corresponds to the second peak.

Despite the amount of the carbon source used (3 or 5 mg g^{-1}), the calculated values of $\Delta_m H$ for each soil were independent of the year of collection and did not significantly differ among the values for each soil. As an average, $\Delta_m H$ was -2654 ± 274 kJ mol^{-1} and -1651 ± 239 kJ mol^{-1} for NYS and YS, respectively. The determined value for NYS is not significantly different from the enthalpy change due to glucose oxidation, $\Delta_{cat} H = -2814.0$ kJ mol^{-1} [19]. On the other hand, the value of $\Delta_m H$ for YS is not significantly different from the aerobic glucose metabolism of *S. cereviciae* of -1976.27 kJ mol^{-1} [20] indicating the prevalence of yeast over the other microorganisms inoculated into the soil.

Fig. 3 shows the trend of SMB and the quotient p/rCO_2 with time during degradation of glucose by the microorganisms contained in YS (Fig. 3A and C) and NYS (Fig. 3B and D). Values of both soils SMB at the different stages of storage at the start of the experiment are not significantly different among them and the average value is SMB = 386 ± 49 μg g^{-1}. However, YS increased its SMB after microbial growth with glucose by $\Delta X = 292 \pm 175$ μg g^{-1} whereas NYS increased by $\Delta X = 396 \pm 117$ μg g^{-1} (see Table 2). Something very interesting is that SMB for the newly recollected soils in 2012 increased by $\Delta X = 778 \pm 83$ μg g^{-1} by the time of the first peak for NYS and the only peak for YS (see Fig. 2). The value of SMB for NYS

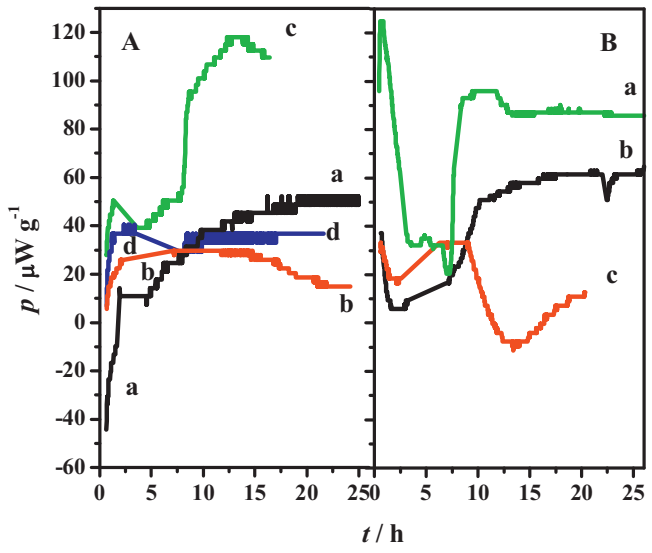

Fig. 1. Mass specific thermal power (p) – time (t) curves of glucose degradation by the microorganisms contained in soil amended with (A) EMA + brewery residual yeast (YS): with (a) 2.0; (b) 3.0 and (c) 5.0 mg g^{-1} glucose (d) with 5.0 mg glucose and 0.5 mg $(NH_4)_2SO_4$ and (B) with EMA (NYS): with (a) 3,0: (b) 5,0 and (c) 50 mg g^{-1} glucose.

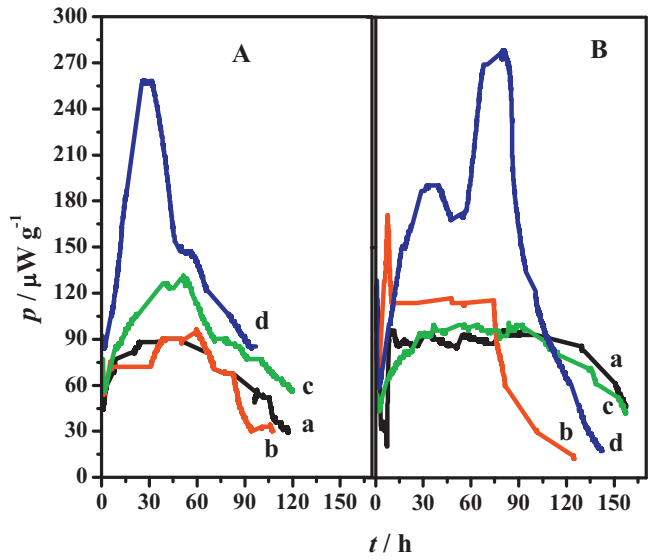

Fig. 2. Specific thermal power (p) – time (t) curves of microbial growth for YS (A) and NYS (B) collected in 2011 amended with (a) 3 mg g^{-1} glucose after 4 months, (b) 3 mg g^{-1} after 7 month, (c) 5 (YS) and 3 mg g^{-1} (NYS) after 12 months from recollection and (d) newly recollected soil in 2012 with 5 mg g^{-1} after 4 months.

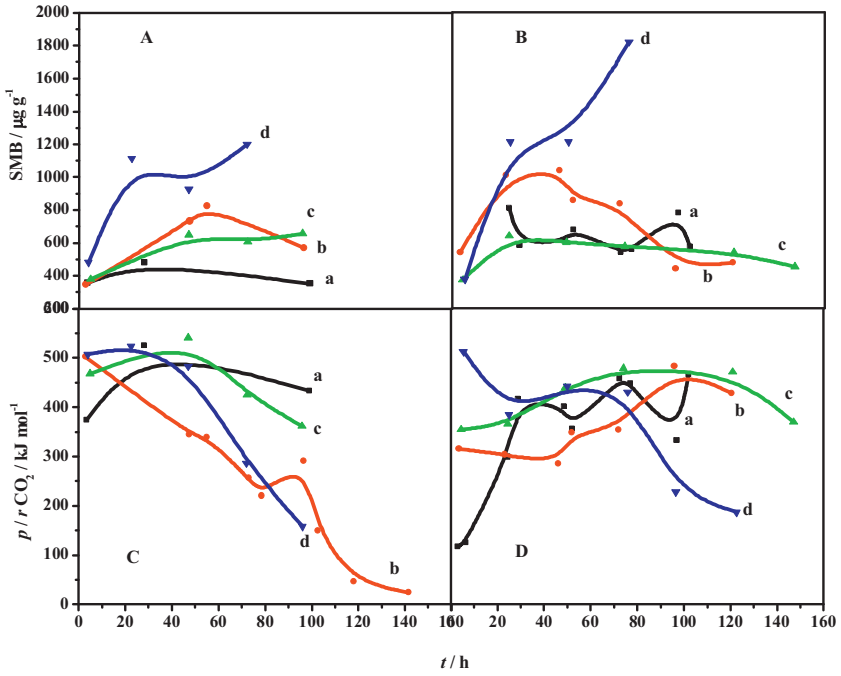

Fig. 3. Soil microbial biomass (A)YS and (B) NYS and calorespirometric ratio (C) YS and (D) NYS of soil recollected in 2011 amended with: (a) 3 mg g^{-1} glucose after 4 months, (b) 3 mg g^{-1} after 7 months, (c) 5 (YS) and 3 mg g^{-1} (NYS) after 12 months and (d) newly collected soil in 2012 with 5 mg g^{-1} after 4 months.

at the time of the second peak (80 h) was around $\Delta X = 608\ \mu g\ g^{-1}$ with respect to the value at the first peak. These results confirm that yeast inhibited some of the soil microbiota or perhaps inhibited the establishment of new microorganisms as occurred with NYS. By using the semilogarithmic equation of exponential microbial growth, the increase in biomass could be calculated and thus, compared with those values determined by direct measurement of CO_2. The increase in biomass, $\Delta X = 787 \pm 70\ \mu g\ g^{-1}$ and $690\ \mu g\ g^{-1}$ calculated for the first (NYS and YS) and second (NYS) peak of newly collected soils, were not significantly different from the directly determined values.

The calorespirometric ratio, p/rCO_2 between the basal specific thermal power and the specific rate of CO_2 evolution of soil without glucose gives a measure of the mineralizing activity of the microorganisms, and equivalent to the metabolic quotient, qCO_2, determined by conventional methods [21]. The calculated values were 335 ± 12 and 199 ± 23 kJ mol^{-1} for YS collected in 2011 and 2012, respectively. Values of p/rCO_2 for NYS were 245 ± 7 in 2011 and 249 ± 67 kJ mol^{-1} in 2012. The latter value is not significantly different from that of YS collected in 2012. Values of p/rCO_2 between 250 and 460 kJ mol^{-1} indicate aerobic degradation of carbohydrates [17] and thus, it is evident that NYS and YS (2012) show high mineralizing activity with low waste of heat (low p). Yeast decreased the mineralizing activity of the soil microbiota in 2011.

Fig. 3C and D show values of p/rCO_2 calculated after the addition of glucose and in this case, these values are a measure of the carbon conversion efficiency of glucose into biomass by the microorganisms contained in the soils. Differences are observed between the soils. The most remarkable is that NYS (Fig. 3D) shows values of p/rCO_2 of 415 ± 49 kJ mol^{-1} during peak time (Fig. 2) whereas the calculated value for YS (Fig. 3C) is 518 ± 25 kJ mol^{-1} indicating again a greater efficiency to convert glucose into biomass by microorganisms of NYS.

A straight line is obtained when values of log SMB are plotted against log p, Fig. 4. Values of SMB were only calculated in those cases where aerobic growth was present, i.e. when values of p/rCO_2 were above 250 kJ mol^{-1}. Values of SMB in curve 'a' of Fig. 4 correspond to p/rCO_2 values above 390 kJ mol^{-1} (> 16 J cm^{-3} CO_2 respired) whereas those values used in curve b of Fig. 4 correspond to p/rCO_2 values between 270 and 390 kJ mol^{-1} (10–16 J cm^{-3} CO_2 respired). Curve 'a' includes the values used to develop the calorimetric method to determine SMB by calorimetry [13]. The slope of 0.899 ± 0.044 is intermediate between those reported for stored and fresh soil (913 and 856, respectively) [22]. By using this relation to determine the increment in SMB, ΔX, a value of $781 \pm 138\ \mu g\ g^{-1}$ was calculated for the first peak and $522\ \mu g\ g^{-1}$ for the second; indicating that calorespirometry is a very useful technique to monitor microbial growth during the whole process.

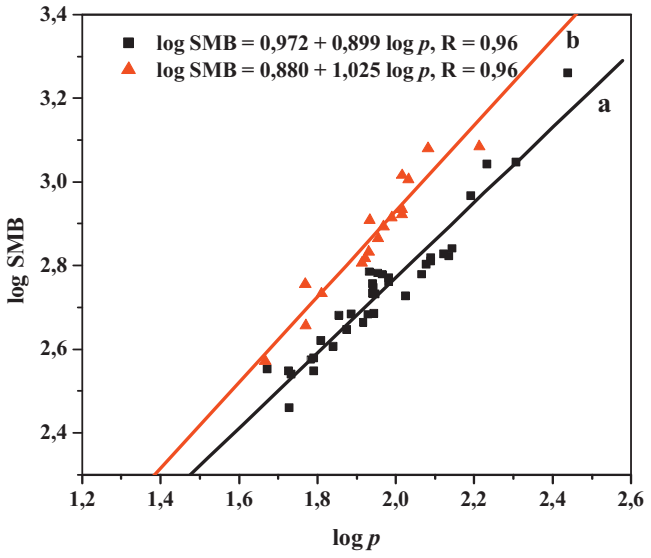

Fig. 4. Plots of log SMB against log p for soils that produced between: (a) 16–22 and (b) 10–16 J cm^3 CO_2 respired.

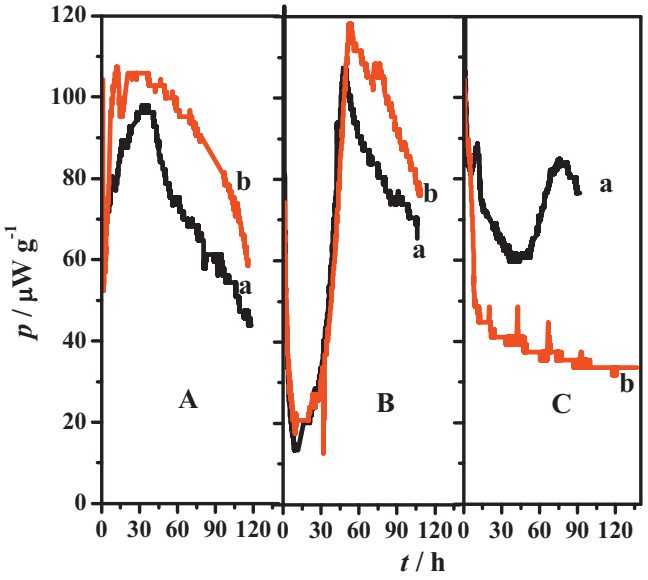

Fig. 5. Specific thermal power – time curves of microbial growth for: (a) YS and (b) NYS collected in 2011 amended with (A) 5 mg g^{-1} sucrose, (B) 5 mg g^{-1} lactic acid and (C) 10 mg g^{-1} cellulose.

It was also interesting to test other carbon substrates, and therefore experiments were performed by using sucrose, lactic acid and cellulose. Fig. 5 shows the $p - t$ curves obtained.

Both soils behaved similar with lactic acid (Fig. 4B: $\Delta_m H = -423 \pm 5$ kJ mol^{-1}, $\Delta_{an} H = 915 \pm 11$ kJ mol^{-1}, 65% anabolic) whereas there was less metabolic energy for microorganisms of YS (Fig. 4A, curve a: $\Delta_m H = -1606$ kJ mol^{-1}, $\Delta_{an} H = 4044$ kJ mol^{-1}, 71% anabolic) toward sucrose than for those of NYS (Fig. 4A, curve b: $\Delta_{met} H = -2585$ kJ mol^{-1}, $\Delta_{an} H = 3065$ kJ mol^{-1}, 54% anabolic). The microflora of these soils spend the same amount of energy to degrade sucrose as to degrade glucose. The soil containing yeast showed activity with cellulose whereas NYS did not.

4. Conclusions

As a conclusion these results show that the brewery yeast had a negative effect on soils. This negative effect subsided after a year from its application even with frequent EMA applications. It was clear that soil microbial activity improved after successive applications of EMA. Interesting result from this work is that it is possible to determine the initial and the increment of SMB by calorespirometry measurements. We have also shown that it is possible to calculate the increment in SMB from a typical calorimetric curve of exponential microbial growth by using the semilogarithmic conversion of the exponential portion and that the result is not significantly different from that determined by calorespirometry.

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